Identification of the Highly Reactive Sulfhydryl Group of Pig Kidney Fructose 1,6-Bisphosphatase at Cysteine 128*

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Fructose 1,6-bisphosphatases contain a highly reactive cysteine residue, the reactivity of which is influenced by ligands that bind at the catalytic and at the allosteric AMP sites of the enzyme. Nevertheless, the sulfhydryl group appears to be proximal to these sites and not a functional component of either. Modification of pig kidney fructose 1,6-bisphosphatase with three reagents, 5,5'-dithiobis-(2-nitrobenzoic acid), iodoacetamide, and phenacyl bromide, yields derivatives with similar properties, thus suggesting that the same residue was modified in each case. The modified enzymes exhibited: (a) higher $V_{max}$ when Mn$^{2+}$ was used as the activating cation; (b) decreased activity in the presence of nonsaturating Mg$^{2+}$ concentrations; (c) no change in sensitivity toward AMP inhibition. Automated Edman degradation of a tryptic peptide containing radioactive carboxamidomethylcysteine showed the sequence of residues Gly-111-Arg-140 of pig kidney fructose 1,6-bisphosphatase. The modified residue was shown to be cysteine-128, and the same cysteine residue was alkylated when the enzyme was reacted with phenacyl bromide. Cysteine-128 is also present in rat and sheep liver fructose 1,6-bisphosphatase and a long stretch of the sequence around this reactive cysteine residue is highly conserved.

Fructose 1,6-bisphosphatase (d-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) is a key enzyme of gluconeogenesis. The native "neutral pH optimum" enzyme isolated from different sources is a tetramer composed of identical subunits of molecular weights ranging from 36,000 to 40,000 (1, 2). A common feature of fructose 1,6-bisphosphatases appears to be the presence of a single highly reactive sulfhydryl group/mol of enzyme subunit (3-7). The reactivity of this cysteine residue toward several sulfhydryl reagents is decreased by the presence of the substrate fructose 1,6-bisphosphate (3, 5, 7), while the allosteric inhibitor AMP markedly increases its reactivity (3, 6, 7). Studies of the properties of several fructose 1,6-bisphosphatases modified at the reactive sulfhydryl group suggest that the cysteine residue is not located at the active or at the allosteric site of the enzyme (3, 4, 7). The reactivity of this group, however, has provided the means to test whether compounds bind at one or the other of these sites in fructose 1,6-bisphosphatases (6, 8, 9). Distance measurements by NMR and EPR relaxation rates show that the reactive cysteine residue is located relatively close to the active site and a model has been proposed to depict the spatial relationships among the catalytic, allosteric, metal ion, and reactive sulfhydryl sites of fructose 1,6-bisphosphatase (10). The present study has established the location of the highly reactive cysteine of pig kidney fructose 1,6-bisphosphatase within the recently determined primary structure of this enzyme (11).

EXPERIMENTAL PROCEDURES AND RESULTS

The amino acid sequence of a 30-residue tryptic peptide isolated from [14C]carboxamidomethyl pig kidney fructose 1,6-bisphosphatase is shown in Fig. 1. This peptide corresponds to the sequence of residues Gly-111-Arg-140 of the enzyme (11) and contains the highly reactive cysteine residue of fructose 1,6-bisphosphatase. This residue has been identified as cysteine-128. The rationale for the methods used to isolate the peptide containing the reactive Cys came from our work on the elucidation of the complete covalent structure of pig kidney fructose 1,6-bisphosphatase (11), a sequence which shows cysteine residues located at positions 38, 92, 116, 128, 183, and 281. S-Carboxamido[14C]methylated fructose 1,6-bisphosphatase labeled only at the reactive cysteine residue was first cleaved with subtilisin into the S-peptide (residues 1-60) and the S-subunit (residues 61-335) components. The highly conserved Cys-38 (22) was ruled out as the reactive sulfhydryl of the enzyme because radioactivity was associated only with the S-subunit. This conclusion is in agreement with our previous observations (14) with NBS-modified enzyme. Analysis of CNBr fragments from the S-subunit (Fig. 6) showed that the radioactivity was associated with...
with the peak of highest molecular weight, corresponding to the peptide containing residues Gly-61-Met-172. This result clearly showed that neither Cys-183, present in the CNBr peak eluting at fractions 203-208, nor Cys-281, present in the CNBr peak eluting at fractions 156-169, contained the carboxamidomethyl label and restricted our search to Cys residues 92, 116, and 128. Finally, the label was located in the tryptic peptide Gly-111-Arg-140 (Fig. 7) and the reactive sulfhydryl was identified by sequence analysis as being that of Cys-128 (Table 1). The identification of this residue as the reactive cysteine explains the presence of the minor radioactive peak eluting at about fraction 138 in the separation of CNBr fragments shown in Fig. 6. Minor cleavage at Asp-Pro peptide bonds occurring during exposure to low pH values such as were encountered in CNBr treatment and peptide separations (23) results in cleavage at Asp-118-Pro-119 leading to the formation of a radioactive fragment Pro-119-Met-182 containing only 1 cysteine residue, the reactive Cys-128.

The region containing the most reactive Cys residue appears to be highly conserved in fructose 1,6-bisphosphatases. Our sequence analysis of this region in rat liver fructose 1,6-bisphosphatase showed identical sequences in the tryptic peptide Gly-111-Arg-140, except for the replacement of Val-130 by asparagine residue (position 32 in the sequence analysis of the pig kidney enzyme by alanine in rat liver fructose 1,6-bisphosphatase). A recent report on the complete sequence of sheep liver fructose 1,6-bisphosphatase (24) reveals full identity from residue 102 through 139 between the sheep liver enzyme and pig kidney fructose 1,6-bisphosphatase. Cysteine-128 is also present in rabbit liver fructose 1,6-bisphosphatase, but in this enzyme Cys-116 present in all other fructose 1,6-bisphosphatases analyzed thus far, has been replaced by an asparagine residue (position 32 in the sequence analysis shown in Fig. 1 in Ref. 25).

Although the identification of the reactive Cys was based upon modification with iodoacetamide, it would appear that the same residue reacts specifically with NBS. The properties of both modified enzymes are rather similar, particularly as regards their response to increasing concentrations of Mn$^{2+}$ in regards to their activity at pH 7.5 measured in the presence of Mn$^{2+}$ was increased, and that measured in the presence of Mg$^{2+}$ was decreased (26, 27). It appears likely therefore that Cys-128 is also the site of reaction with dinitrofluorobenzene, although this has not been proven as yet.

The specific reaction of Cys-128 of fructose 1,6-bisphosphatase with phenacyl bromide may permit the exploration of the environment of that cysteine residue using the related bifunctional reagent, $p$-azidophenacyl bromide (28). Indeed, preliminary experiments in our laboratory indicate that modification of fructose 1,6-bisphosphatase with this reagent produces a derivative with properties similar to those of the phenacyl bromide-modified enzyme. Irradiation of the covalently bound reagent should give a highly reactive nitrene likely to generate cross-links in the vicinity of the catalytic and allosteric sites of the enzyme. Analysis of the cross-linked region may provide three-dimensional structure information for fructose 1,6-bisphosphatases based upon chemical modification. Cysteine 128 also appears to react rapidly and specifically with either $p$-hydroxymercuribenzoate or $p$-hydroxymercuriphenylsulfonate, thus opening possibilities for preparing heavy metal Cys-128 derivatives of fructose 1,6-bisphosphatase suitable for x-ray diffraction. These experiments are now in progress.

Acknowledgment—We thank Dr. B. L. Horecker for providing us with unpublished information on the amino acid sequence of rabbit liver fructose 1,6-bisphosphatase.

REFERENCES

The Reactive Sulphydryl of Fructose 1,6-Bisphosphatase

IDENTIFICATION OF THE HIGHLY REACTIVE SULFHYDRYL GROUP OF PIG KIDNEY FRUCTOSE 1,6-BISPHOSPHATASE AT CYSTEINE-128

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EXPERIMENTAL PROCEDURES

Identification of the Reactive Sulphydryl

In a typical experiment, the reaction mixture (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 10% glycerol) was incubated at 30 °C for 20 min. The enzyme was then dialyzed against 100 mM Tris-HCl, pH 8.5, 500 mM KCl, and 25% glycerol for 24 h at 4 °C. A 5% (w/v) solution of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) (20 mM) was added to the enzyme solution to a final concentration of 400 mM. The reaction was initiated by the addition of 100 µM fructose 1,6-bisphosphate (FBP) to give a final concentration of 1 mM. The reaction was stopped after 2 min by the addition of 400 mM DTNB. The reaction was then stopped by the addition of 400 mM DTNB. The absorbance at 412 nm was measured to determine the extent of the reaction.

Table 1

<table>
<thead>
<tr>
<th>Cycle Number</th>
<th>Major T4 Deficiency</th>
<th>Relative Activity (%)</th>
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<tr>
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<tr>
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The assay of the relative activity was performed as a control and for the determination of the T4 deficiency. The results were expressed as a percentage of the activity of the major T4 deficient enzyme.

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