Probing the pyrophosphate-binding site in potato tuber UDP-glucose pyrophosphorylase with pyridoxal diphosphate

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Abstract

Potato tuber UDP-glucose pyrophosphorylase (EC 2.7.7.9) catalyzes the reversible uridylyl transfer from UDP-glucose to MgPP_i forming glucose 1-phosphate and MgUTP, according to an ordered bi-bi mechanism in which UDP-glucose and MgPP_i bind in this order. To probe the active site of this enzyme, we have applied pyridoxal 5'-diphosphate, a reactive PP_i analogue. The enzyme was rapidly inactivated when incubated with the reagent in the presence of Mg²⁺ followed by sodium borohydride reduction. The degree of the inactivation was decreased by MgUTP, MgPP_i, and glucose 1-phosphate, but enhanced by UDP-glucose. The enhancement was prevented by co-addition of P_i, the competitive inhibitor with respect to PP_i. The complete inactivation corresponded to the incorporation of 0.9–1.1 mol of reagent/mol of enzyme monomer. In the presence of UDP-glucose, labels were almost exclusively incorporated into Lys-329. Thus, this residue may be located near the bound MgPP_i and its modification is promoted, probably through conformational changes, by the binding of UDP-glucose to the enzyme. The results of the modification by the same reagent of the mutant enzymes in which Lys-329 and Lys-263 are individually replaced by Gln suggest the roles of these lysyl residues in the binding of MgPP_i and in the UDP-glucose-induced conformational changes, respectively.

Keywords: active site; affinity labeling; PP_i-binding site; pyridoxal 5'-diphosphate; UDP-glucose pyrophosphorylase

UDP-glucose pyrophosphorylase (EC 2.7.7.9) from potato tuber is a 52-kDa monomeric enzyme that catalyzes the reversible transfer of a uridylyl group from UDP-glucose to MgPP_i forming glucose-1-P and MgUTP. The reaction proceeds by an ordered bi-bi mechanism in which UDP-glucose and MgPP_i are bound and glucose-1-P and MgUTP are released in this order (Nakano et al., 1989). The primary structure of this enzyme was deduced from the cDNA nucleotide sequence (Katsube et al., 1990). An expression plasmid for efficient production of the enzyme in *Escherichia coli* was also constructed. The recombinant enzyme purified was structurally and catalytically iden-

tical with the natural enzyme except for the absence of an N-terminal-blocking acetyl group (Katsube et al., 1991).

To probe the active site of this enzyme, we have used reactive analogues of UDP-glucose, uridine di- and triphosphopyridoxals, and pyridoxal(5')diphospho- α -D-glucose (Kazuta et al., 1991a,b). These compounds similarly inactivated the enzyme in a stoichiometric manner and labeled the identical set of five lysyl residues (Lys-263, Lys-329, Lys-367, Lys-409, and Lys-410). These results suggested the presence of five lysyl residues at or near the UDP-glucose (or UTP)-binding site (Kazuta et al., 1991a). Interestingly enough, the amounts of analogues incorporated into each lysyl residue differed depending on the reagents used and the presence or absence of substrate, even though about half of the analogues were incorporated into Lys-367 in every case. Based on comparison of the incorporation of analogues into each lysyl residue (comparative affinity labeling), we have presented a hypothetical model for the location of those residues around the substrate bound to the enzyme (Kazuta et al., 1991b). In addition, we have replaced the five lysyl residues individ-

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Abbreviations: PLDP, pyridoxal 5'-diphosphate; glucose-1-P, α -D-glucose 1-phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography. For mutant enzymes: for example, Lys-329/Gln represents the mutant enzyme in which Lys-329 is replaced by Gln.

ually by glutamine via site-directed mutagenesis to examine their functional roles (Katsube et al., 1991). Among those residues, Lys-367 was found to be essential for the catalytic activity of this enzyme, the mutant enzyme, in which Lys-367 was replaced, being almost completely inactive. The kinetic properties of the mutant enzymes were consistent with the hypothetical model constructed in the comparative affinity-labeling study.

To study in more detail the active site of this enzyme, we have presently used a reactive PP_i analogue, PLDP. The reagent is effectively bound, in the presence of Mg^{2+} , to the PP_i -binding site in the enzyme-UDP-glucose complex. The reagent labels mainly Lys-329. The results of the modification with MgPLDP of the mutant enzymes in which Lys-329 and Lys-263 are individually replaced by Gln suggest the roles of Lys-329 and Lys-263 in the binding of $MgPP_i$ and the UDP-glucose-induced conformational changes, respectively.

Results

Inactivation by PLDP

Incubation of recombinant potato tuber UDP-glucose pyrophosphorylase with PLDP at different concentrations in 50 mM HEPES-NaOH (pH 8.0) followed by sodium borohydride reduction resulted in dose- and time-dependent inactivation of the enzyme (Fig. 1a). The absorbance at 430 nm in difference spectra between the enzyme plus PLDP and the reagent alone (without sodium borohydride reduction) increased in parallel with the inactivation, suggesting the formation of a Schiff base (Shapiro et al., 1968). Cessation of the inactivation after a certain period of time is typical for the modification by reagents containing a pyridoxal moiety (e.g., Kazuta et al., 1991a) and is due to the fact that the Schiff base is in equilibrium with a noncovalently bound form of the reagent. Only the enzyme forming a Schiff base is irreversibly labeled and inactivated by borohydride reduction.

Addition of MgUTP, glucose-1-P, or $MgPP_i$ to the reaction mixtures retarded to different degrees the inactivation of the enzyme by PLDP (Fig. 1b), as previously observed in the modification with other affinity-labeling reagents (Kazuta et al., 1991a,b). In contrast, UDP-glucose affected the inactivation differently. The inactivation by PLDP was markedly promoted by addition of UDP-glucose. Similar compounds, having different numbers of phosphate groups, pyridoxal 5'-monophosphate, and pyridoxal 5'-triphosphate, also inactivated the enzyme, but the inactivations by these compounds were, unlike that by PLDP, retarded by UDP-glucose (data not shown).

Promotion of inactivation

The promotion by UDP-glucose of the inactivation was observed at any concentration (3–20 μ M) of PLDP but

Fig. 1. Inactivation of potato tuber UDP-glucose pyrophosphorylase by MgPLDP. **a:** Dose-dependency. The inactivation mixtures (200 μ L) containing 50 mM HEPES (pH 8.0), 104 μ g of enzyme, 5 mM $MgCl_2$, and PLDP at 3 μ M (○), 5 μ M (●), 7 μ M (□), 10 μ M (△), or 20 μ M (■) were incubated at 25 °C. At the indicated times, aliquots were withdrawn, reduced with 5 mM sodium borohydride, and diluted with 25 mM Tris-HCl (pH 8.0), and the residual activity was assayed. **b:** Effect of substrates. The inactivation mixtures (200 μ L) containing 50 mM HEPES (pH 8.0), 104 μ g of enzyme, 5 mM $MgCl_2$, and 20 μ M reagent plus none (●), 2 mM UDP-glucose (○), 2 mM UTP (■), 2 mM glucose-1-P (□), or 2 mM PP_i (▲) were incubated at 25 °C. At the indicated times, aliquots were withdrawn, reduced with 5 mM sodium borohydride, and diluted with 25 mM Tris-HCl (pH 8.0), and the residual activity was assayed.

did not occur in the absence of Mg^{2+} . Among other metal ions tested, only Mn^{2+} could replace Mg^{2+} . The degree of promotion increased depending on the concentration of Mg^{2+} below 50 μ M and was saturated at this concentration. The promotion was also dependent on the concentration of UDP-glucose, reaching a maximum around 0.1 mM (Fig. 2). Both UDP- and ADP-glucose act as competitive inhibitors with respect to UDP-glucose ($K_i = 0.5$ mM and 4 mM, respectively). The addition of UDP- or ADP-glucose caused retardation, not promotion, of the inactivation by PLDP. ADP-glucose appeared to be less effective in the retardation of the inactivation than UDP. This may be attributable to different K_i values of two compounds. Indeed, when they were added separately up to 10 mM, similar residual activities (about 65%) were obtained (data not shown).

To elucidate where MgPLDP is bound in the presence of UDP-glucose, the protective effect of P_i , a competitive inhibitor ($K_i = 18$ mM) with respect to PP_i , was investigated. PP_i could not be used together with UDP-glucose

Fig. 2. Effect of UDP-glucose, UDP, and ADP-glucose on the inactivation of potato tuber UDP-glucose pyrophosphorylase by MgPLDP. The inactivation mixtures (200 μ L) containing 50 mM HEPES (pH 8.0), 104 μ g of enzyme, 5 mM MgCl₂, and 20 μ M PLDP plus indicated concentrations of UDP-glucose (○), UDP (□), or ADP-glucose (●) were incubated at 25 °C for 10 min. Aliquots were withdrawn, reduced with 5 mM sodium borohydride, and diluted with 25 mM Tris-HCl (pH 8.0), and the residual activity was assayed.

because the enzyme reaction would occur by its addition. Not only the promotion was diminished but also the inactivation itself was retarded by addition of P_i in a dose-dependent manner (Fig. 3). The half maximal retardation by P_i of the enhanced inactivation was achieved around 20 mM, similar to the *K_i* value for P_i.

Fig. 3. Effect of P_i on the UDP-glucose-enhanced inactivation of potato tuber UDP-glucose pyrophosphorylase by MgPLDP. The inactivation mixtures (200 μ L) containing 50 mM HEPES (pH 8.0), 104 μ g of enzyme, 2 mM UDP-glucose, 5 mM MgCl₂, and 20 μ M PLDP plus indicated concentration of sodium phosphate were incubated at 25 °C for 10 min. Aliquots were withdrawn, reduced with 5 mM sodium borohydride, and diluted with 25 mM Tris-HCl (pH 8.0), and the residual activity was assayed.

Stoichiometry of inactivation

To determine stoichiometry of the inactivation with PLDP, the enzyme was incubated with 20 μ M PLDP in the absence or presence of 2 mM UDP-glucose at 25 °C for 0.25, 0.5, 1, 2, 3, and 5 min. The inactivation was terminated by addition of sodium borohydride. The reaction mixtures were applied to gel filtration columns, and fluorescence derived from the pyridoxyllysine moiety (excitation at 320 nm; emission at 390 nm), residual activities, and protein concentrations of passed-through fractions were measured. The plot of residual activity against the amount of bound reagent gave a straight line in both absence and presence of UDP-glucose (Fig. 4). Extrapolations of the lines to zero activity yield values of 0.9 mol and 1.1 mol of covalently incorporated PLDP per mol of enzyme monomer in the absence and presence of UDP-glucose, respectively.

Identification of the PLDP-labeled site

To identify the site(s) labeled by MgPLDP, the enzyme was modified with 20 μ M PLDP in the absence or presence of 2 mM UDP-glucose, followed by carboxymethylation, and then cleaved by lysyl endopeptidase. The digests were separated by HPLC as described in the Materials and methods. Fluorescent peptides from the enzyme labeled in the absence of UDP-glucose were eluted as more than six peaks (Fig. 5a). In the elution pattern of peptides from the enzyme labeled in the presence of the substrate, two peaks increased remarkably, whereas the remainder almost disappeared (Fig. 5b). The two increased peaks (termed peaks A and B in the order of elution) were separately pooled and lyophilized. To remove the phosphate moiety in the bound reagents, the labeled peptides were treated with pyrophosphatase and alkaline

Fig. 4. Stoichiometry of inactivation of potato tuber UDP-glucose pyrophosphorylase by MgPLDP in the absence (○) or presence of UDP-glucose (●). See the text for experimental procedures.

Fig. 5. High-performance liquid chromatography profiles of the lysyl endopeptidase digest of potato UDP-glucose pyrophosphorylase labeled by MgPLDP in the absence (a) or presence (b) of UDP-glucose. See the text for experimental procedures.

phosphatase as described in the Materials and methods. Without this treatment, rechromatography of the labeled peptide using a C18 reverse-phase column was difficult, resulting in diverse peaks.

After the phosphatase treatment, peaks A and B were eluted from C18 columns at the same retention time. Two peptides in these peaks had the same amino acid compositions. Sequence analyses revealed an identical structure: Met-Glu-Ile-Ile-Pro-Asn-Pro-X-Glu-Val-Asp-Gly-Val-Lys. This sequence corresponds to that from Met-322 to Lys-335, except for Lys-329, in the amino acid sequence of potato UDP-glucose pyrophosphorylase (Katsube et al., 1990). Thus, we conclude that the main MgPLDP-labeled site is Lys-329, whereas, in the absence of UDP-glucose, many other lysyl residues besides Lys-329 are also labeled.

The reason why a single peptide behaved differently in the first chromatography is unknown; however, this may be attributable to the equilibrium between differently dissociated forms of the diphosphate moiety of the reagent. Because more than 85% of the fluorescence (calculated from the areas of peaks) was eluted as peaks A and B, MgPLDP almost exclusively labeled Lys-329 of the enzyme in the presence of UDP-glucose.

Labeling of mutant enzymes

Five mutant enzymes of potato tuber UDP-glucose pyrophosphorylase were prepared by the individual replacement by glutamine of the five lysyl residues located at or near the UDP-glucose (or UTP)-binding site (Katsube et al., 1991). We have tested each mutant enzyme for the modification with MgPLDP. The mutant enzymes purified showing varieties of enzyme activities were incubated

with 5 mM $MgCl_2$ and 20 μM PLDP, in the absence or presence of 2 mM UDP-glucose at 25 °C for 10 min. After addition of 5 mM sodium borohydride, residual activities were measured.

In the absence of UDP-glucose, all of the mutant enzymes were effectively inactivated to residual activities varying from 40 to 70% of the original activities (Fig. 6). The mutant enzymes were inactivated by modifications of lysyl residues other than the target lysine that had been replaced by inert glutamine. The variation in residual activities appears to be related to that of the original activities of the mutant enzymes.

Addition of UDP-glucose enhanced the degree of the inactivation by MgPLDP of three mutant enzymes (Lys-367/Gln, Lys-409/Gln, and Lys-410/Gln), similar to the wild-type enzyme. In contrast, the same addition retarded the inactivation of the Lys-329/Gln mutant enzyme. As shown in the preceding section, MgPLDP specifically labels Lys-329 of the wild-type enzyme in the presence of UDP-glucose, whereas, in its absence, many lysyl residues including Lys-329 are labeled. The Lys-329/Gln mutant enzyme was only slightly inactivated with MgPLDP in the presence of UDP-glucose, since it has no more lysine at position 329. However, in the absence of UDP-glucose, the same mutant enzyme was significantly inactivated through modifications of lysyl residues other than Lys-329.

The degree of the inactivation of the Lys-263/Gln mutant enzyme was also reduced by addition of UDP-glucose though slightly. To determine the labeled site in the Lys-263/Gln mutant enzyme, the mutant enzymes that had been labeled by MgPLDP in the absence and presence of UDP-glucose were digested by lysyl endopepti-

Fig. 6. Inactivation of mutant enzymes of potato tuber UDP-glucose pyrophosphorylase by MgPLDP in the presence or absence of UDP-glucose. See the text for experimental procedures. The original specific activities of wild-type and mutant enzymes measured at standard assay conditions were as follows: wild-type, 1,300 units/mg; Lys-263/Gln, 16 units/mg; Lys-329/Gln, 850 units/mg; Lys-367/Gln, 0.12 units/mg; Lys-409/Gln, 910 units/mg; Lys-410/Gln, 1,060 units/mg. Unshaded and shaded bars represent residual activities of the enzyme inactivated in the absence and presence of UDP-glucose, respectively.

dase. The resultant peptides were chromatographed on a C4 reverse-phase column and two chromatographic patterns were compared. Addition of UDP-glucose only slightly increased two peaks corresponding to peptides A and B (equivalent to the amount of label incorporated into Lys-329) but almost completely diminished all other small peaks (data not shown).

Discussion

Pyridoxal 5'-phosphate has been used to modify lysyl residues in various proteins, though the modifications are only partially specific for sites. To increase the specificity for the nucleotide-binding site, a nucleotidyl group was coupled to pyridoxal 5'-phosphate through a pyrophosphate linkage. The syntheses of such reagents, uridine diphosphopyridoxal (Tagaya et al., 1985) and adenosine diphosphopyridoxal (Tagaya & Fukui, 1986; Tamura et al., 1986), were reported independently by two laboratories. Since then, reagents of this type with different bases and/or different numbers of the phosphate moiety have been successfully applied for affinity labeling of many proteins. For potato tuber UDP-glucose pyrophosphorylase, uridine di- and triphosphopyridoxals, and pyridoxal(5')diphospho- α -D-glucose have been used to probe the substrate-binding site (Kazuta et al., 1991a,b). The results of those affinity labeling studies suggested the presence of five lysyl residues at or near the UDP-glucose (or UTP)-binding site. A hypothetical model for the location of those residues around the bound substrates was presented based on the comparison of the amounts of labels incorporated into each residue.

Presently, we have compared pyridoxal 5'-mono-, di-, and triphosphates as affinity labels for modification of potato tuber UDP-glucose pyrophosphorylase. The three reagents effectively inactivated the enzyme but the degree of only the inactivation by MgPLDP was enhanced by addition of UDP-glucose (Figs. 1, 2). This finding is understandable by assuming that MgPLDP acts as an affinity label binding specifically to the MgPP_i-binding locus at the active site of the enzyme, because the binding of MgPP_i to the enzyme follows UDP-glucose binding (Nakano et al., 1989). This assumption is supported by the finding that the inactivation was markedly retarded by addition of P_i, a competitive inhibitor with respect to PP_i (Fig. 3). The complete inactivation corresponded to the incorporation of about 1 mol of reagent per mol of enzyme monomer (Fig. 4). Actually, MgPLDP in the presence of UDP-glucose mainly labeled Lys-329. Therefore, we suggest that MgPLDP acts as a reactive analogue of MgPP_i binding to the specific locus in the enzyme. Binding of UDP-glucose increases, probably through conformational changes of protein, the specificity of the reagent to Lys-329. Affinity labeling reagents effective for PP_i-binding locus have never been reported, and MgPLDP might be applicable also to other proteins to probe the PP_i-binding site.

Tamura et al. (1988) demonstrated that glucose enhanced the degree of the inactivation of yeast hexokinase PII by adenosine diphosphopyridoxal that was suggested to bind to the site specific for ATP. In an open conformation, a deep central cleft divides two lobes in each subunit of this enzyme. Upon binding of glucose, the smaller lobe containing a lysyl residue rotates partially, closing the cleft (Bennett & Steitz, 1978). The movement of positive charges into the central cleft might be a common step in the tight binding of nucleotides by bilobal kinases (Ladine & Cross, 1991). Based on the present observations, we presume that conformational changes similar to those found in kinases occur also in potato tuber UDP-glucose pyrophosphorylase upon binding of UDP-glucose. To prove this hypothesis, X-ray crystallographic analyses on the three-dimensional structures of this enzyme in its free and complex forms are in progress by us collaborating with another group at Osaka University.

The sequence analysis of the peptide labeled with MgPLDP in the presence of UDP-glucose identified Lys-329 as the labeled site. This residue was also labeled with reactive analogues of UDP-glucose although the amount of label incorporated into this residue is not high (Kazuta et al., 1991a,b). The inactivation by those reagents was almost completely prevented by addition of UTP or UDP-glucose. UTP also retarded the incorporation of any reagent into Lys-329. However, UDP-glucose increased the relative amount of incorporation of uridine triphosphopyridoxal into this lysyl residue. Thus, Lys-329 was tentatively placed near the PP_i bound to the active site (Kazuta et al., 1991b). This conclusion was consistent with the results of mutagenesis studies. The mutation of Lys-329 to Gln resulted in a 22-fold increase in K_m for MgPP_i even though K_m for other substrates as well as V_{max} did not change drastically (Katsube et al., 1991). K_m for MgPP_i was also increased by the mutation of Lys-263 to glutamine, which resulted in a decrease by two orders of magnitude in V_{max} .

The degree of the inactivation by MgPLDP of only two mutant enzymes (Lys-329/Gln and Lys-263/Gln) was decreased by addition of UDP-glucose, whereas that of the other mutant enzymes was enhanced by the same addition (Fig. 6). As shown already, MgPLDP mainly labeled Lys-329 of the wild-type enzyme in the presence of UDP-glucose, whereas, in its absence, the reagent labels numbers of lysyl residues. It is therefore reasonable that the Lys-329/Gln mutant enzyme is less labeled by MgPLDP in the presence of UDP-glucose than its absence. The replacement of Lys-263 by Gln significantly increased K_m values for PP_i and glucose-1-P (Katsube et al., 1991). Based on the results of comparative affinity-labeling studies, Lys-263 was tentatively located near the glucose phosphate moiety of UDP-glucose (Kazuta et al., 1991b). Presently, it has been shown that the Lys-263/Gln mutant enzyme lost the ability to enhance the reactivity of Lys-329 for labeling by MgPLDP. Thus, this residue may be

involved in the UDP-glucose-induced conformational changes rather than the direct interaction with MgPP_i . We presume roles for Lys-263 and Lys-329 in the UDP-glucose-induced conformational changes and the direct interaction with MgPP_i in the enzyme-UDP-glucose complex, respectively.

Materials and methods

Reagents

Phosphoglucomutase (rabbit muscle), glucose-1-P, and nucleotide pyrophosphatase (*Crotalus adamanteus* venom) were obtained from Sigma Chemical Co. NADP^+ was purchased from Kojin. UDP-glucose and pyridoxal 5'-phosphate were purchased from Nacalai Tesque. Glucose-6-phosphate dehydrogenase was obtained from Oriental Yeast. HEPES was obtained from Dojin. Lysyl endopeptidase and sodium pyrophosphate were from Wako Pure Chemicals. Calf intestine alkaline phosphatase was purchased from Takara Shuzo. Pyridoxal 5'-di- and triphosphates were synthesized according to the method of Shimomura and Fukui (1978) and Tagaya et al. (1987), respectively.

Purification of wild-type and mutant UDP-glucose pyrophosphorylase

Purification of recombinant potato UDP-glucose pyrophosphorylase and preparations of mutant enzymes were described previously (Katsube et al., 1991). Before use, the enzyme was passed through a column of Sephadex G-25 that had been equilibrated with 25 mM HEPES (pH 8.0). The concentration of the enzyme was spectrophotometrically determined by using the absorbance at 280 nm at 1 mg/mL of 0.49 (Nakano et al., 1989). A molecular weight of 51,900 (Katsube et al., 1990) was used for calculation.

Enzyme assay

The assay mixture (0.74 mL) contained 50 mM Tris-HCl (pH 8.0), 7 mM MgCl_2 , 2 mM UDP-glucose, 5 mM PP_i , 20 μM glucose 1,6-bisphosphate, 0.3 mM NADP^+ , and 1 unit each of phosphoglucomutase and glucose-6-phosphate dehydrogenase. Assay was started by addition of PP_i , and the increase in the absorbance at 340 nm was continuously monitored with a spectrophotometer at 30 °C. One unit of the enzyme activity was defined as the amount of enzyme that produced 1 μmol of glucose-1-P per minute under the above conditions.

Isolation of labeled peptides

A recombinant potato UDP-glucose pyrophosphorylase solution (10 μM , 6 mL) was incubated with 20 μM PLDP

in the presence of 5 mM MgCl_2 and 2 mM UDP-glucose at 25 °C for 10 min. The reaction was stopped by adding a freshly prepared solution of NaBH_4 to a final concentration of 5 mM. The solution was kept at 0 °C for 30 min. The labeled enzyme thus prepared was carboxymethylated, extensively dialyzed, and lyophilized.

The labeled enzyme was suspended in 150 μL of 0.1 M Tris-HCl (pH 7.5) containing 8 M urea, and then 150 μL of 0.1 M Tris-HCl (pH 7.5) was added. Lysyl endopeptidase was added to the turbid solution in a 1:50 (mol/mol) ratio of protease to substrate. Digestion was performed at 36 °C for 12 h and then terminated by immersing the reaction vessel in boiling water for 3 min. Labeled peptides were separated on a Gilson HPLC system equipped with a Vydac C4 reverse-phase column at a flow rate of 1.0 mL/min. The solvent used was 0.1% trifluoroacetic acid (A) and 0.095% trifluoroacetic acid containing 90% acetonitrile (B). The absorbance at 215 nm and the fluorescence (excitation at 320 nm and emission at 390 nm) of effluents were continuously monitored.

Phosphatase treatment of labeled peptide

The fluorescent materials lyophilized were dissolved in 300 μL of 0.5 M Tris-HCl (pH 7.7) containing 5 mM MgCl_2 , and then 5 units of nucleotide pyrophosphatase and 20 units of calf intestine alkaline phosphatase were added. Reaction was performed at 36 °C for 10 h and then terminated by immersing the reaction vessel in boiling water for 5 min. After discarding insoluble materials, the labeled peptide was separated on HPLC equipped with a Vydac C18 reverse-phase column.

Amino acid composition and sequence analysis

Amino acid composition was determined, after hydrolysis with 6 N HCl in an evacuated tube, on a Hitachi 835 amino acid analyzer using *o*-phthalaldehyde. Proline was not detectable in this system. Amino acid sequence was determined with an Applied Biosystems model 477A protein sequencer linked with an Applied Biosystems model 120A PTH analyzer.

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