Active Site Lysine Promotes Catalytic Function of Pyridoxal 5'-Phosphate in α-Glucan Phosphorylases*

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The activity of α-glucan phosphorylases (EC 2.4.1.1) is dependent on pyridoxal 5'-phosphate (pyridoxal-P) which, in carbohydrate phosphorylation, acts quite differently from its role in other pyridoxal-P dependent enzymes. Numerous studies with pyridoxal-P derivatives, 31P NMR spectroscopy, and with a new class of “glycosylc” substrate analogs demonstrated that not the formation of a Schiff base intermediate, but close contacts to the pyridoxal-P 5'-phosphate to the substrate phosphate is indispensable for activity (for review see Refs. 1–3). Hydrogen bond contacts enable the 5'-phosphate to act reversibly as a proton donor with the substrate phosphate as acceptor and in catalysis, most probably in maintaining the electrostatic neutrality of the pyridoxal 5'-phosphate and aligning the close phosphate-phosphate contacts indispensable for the proton transfer mechanism.

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X-ray crystallography of the phosphorylase b-heptulose 2-phosphate complex revealed a cluster of positively charged amino acids around the active site (4). Among those, only Lys563 is in hydrogen bond distance to the 5'-phosphate of pyridoxal-P, but there are no direct hydrogen bonds of Lys563 to the substrate phosphate or the sugar-phosphate (Fig. 1). Since glycogen phosphorylases are highly conserved in eu- and prokaryotes (5) and the amino acid sequences at the catalytic site are essentially identical (6), the role of the corresponding Lys563 in the unregulated maltodextrin phosphorylase was probed by site-directed mutagenesis. In the present study different kinetic properties of the Lys563Ser and the Lys563Gln maltodextrin phosphorylase mutants are reported and a structure-function relationship for Lys563 is suggested.

MATERIALS AND METHODS

Strains and Plasmids—Escherichia coli strain pop2158 [m1A518, F', araD139, lacU169, rpsL, relA, thiA] was kindly provided by Dr. M. Schwartz, Institut Pasteur, Paris. Plasmid pMAP101 was constructed from plasmid pOM13 as described before (7, 8). The strains used for mutagenesis were a gift from Dr. H. J. Fritz, Göttingen: E. coli BMH71-18[lac-proAB, thi, supE, lacP, lacZΔM15, proA'B'], BMH71-18mutS:[BMH71-18 mutS215:ThiJ], MK30-3:[lac-proAB, recA, galE, strA, F', lacP, ΔM15, proA'B'] and phage M13mp8.

DNA Procedures—DNA was manipulated by standard procedures (9). Restriction endonucleases, T4 DNA-ligase, and polynucleotide kinase were used as recommended by the manufacturer (Boehringer Mannheim). Transformation of E. coli was performed by the method of Hanahan (10). Oligonucleotides were synthesized on an Applied Biosystem 380A DNA synthesizer and purified by preparative electrophoresis on a denaturing 20% polyacrylamide gel.

Mutagenesis—Site-directed mutagenesis was accomplished by the gapped duplex approach (11) on the closed madP as described (12) with a 22-base pair oligonucleotide “ATTGACATCTGTTGCACG” in which the AAA codon for Lys563 was replaced by a TCA codon for Ser. This exchange, in addition, created a BglII recognition site to facilitate screening. The Lys563Gln substitution was introduced using a 21-base pair oligonucleotide “TATTGACATCTGTTGCACG” changing the AAA codon to a CAA codon for Gln. Mutants were identified by sequencing for an additional XhoII site created by the oligonucleotide used for mutagenesis. To ensure that no secondary priming site existed, the same oligonucleotide was used as a sequencing primer. Only one priming site was found. The exchanges were confirmed by sequencing.

Enzyme Purification—E. coli pop2158 carrying plasmid pMAP101 or mutagenized derivatives were grown in Luria broth medium supplemented with 0.4% maltose for 24 h. Washed cells were disrupted by sonication or by repeated passage through a French press. The cell-free extracts of mutant enzymes were further purified as described for mutant and wild-type enzyme (12, 13). Purification of low-activity mutant proteins was followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Pyridoxal-P content of the protein was determined as described by Wada and Snell (14). Protein concentration was measured chemically (15) or from the absorbance at 280 nm, using E1%/1% = 1.36.

Kinetic Measurements—Enzyme activity was assayed at 30°C in the direction of phosphorylase in a coupled assay (16) in 50 mM Tris acetate or MES buffer, pH 6.9, containing 10 mM maltotetraose (generously provided by Boehringer Mannheim) or 2% dextrin, 10 mM KH₂PO₄, 1 mM NAD⁺, 5 mM MgCl₂, 1 mM EDTA, 5 μM glucose-1,6-bisphosphate, 2 μg/ml phosphoglucomutase, and 2 μg/ml Leuconostoc mesenteroides glucose-6-P dehydrogenase. In the direction of synthesis the enzyme was assayed in 25 mM MES buffer, pH 6.9, 10 mM Glc-1-P, and 5 mM maltotetraose. The release of P was measured by the method of Saheki et al. (17). Initial velocity studies with low-

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1 The abbreviations used are: pyridoxal-P, pyridoxal 5'-phosphate; MES, 2-morpholinooethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
concentrations of cations (≤20 mM) were performed at 30 °C by varying the concentration of the substrates within a 10-fold range on a Beckmann DU64 spectrophotometer. No differences in activity was observed for several buffers used (MES, HEPES, glycophosphatase buffer). Since the enzymes of the coupled assay are sensitive to high concentrations of cations the assay described above was modified: An assay containing 10 mM maltoheptaose, varying concentrations of phosphate (as Tris phosphate, pH 6.9) and cations was adjusted to 50 mM Tris with Tris acetate, pH 6.9. Aliquots were drawn at 30 °C at different times and heat inactivated (5 min at 70 °C). The denatured protein was removed by a short centrifugation step (3 min in an Eppendorf centrifuge), and the Glc-1-P concentration was determined to be about 80 mM, the kinetic parameters were determined by nonlinear regression (20).

**RESULTS AND DISCUSSION**

Ly8393 was changed to a serine or a glutamine by the gapped duplex approach of site-directed mutagenesis (11) on a BglII-PstI fragment of the E. coli malP gene cloned into M13mp9rev. Both mutant and wild-type enzymes exhibited a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an apparent molecular weight of 90,000. Binding to glycogen-Sepharose showed that substrate affinity was retained in both mutants. The cofactor content was determined to be 1 mol pyridoxal-P/mol of subunit for the mutant enzyme, therefore, stoichiometry of pyridoxal-P is retained. The possibility that the remaining activity is due to a contamination with traces of wild-type enzyme is ruled out for the Ly8393-Ser mutant by the difference in $K_m$ values and the dependence of mutant activity on small cations. Furthermore, both mutants differ from the wild-type enzyme in the capability to use the glycolytic substrate analog glucal. Maltodextrin phosphorylase catalyze the formation of 2-deoxyglucosyl-(α-1,4-glucoside), from D-glucal and maltotetraose in the presence of phosphate, for the reverse reaction three products were identified: maltotetraose, 2′-deoxyglucose 1-P, and D-glucal. In the equivalent assay, both mutants of Ly8393 yield 2-deoxyglucose 1-P and maltotetraose but fail to produce D-glucal from 2′-deoxyglucose 1-(α-1,4-glucoside). Since no formation of D-glucal was observed, a contamination of more than one wild-type enzyme molecule/10,000 mutant molecules can be ruled out, this property was used to test for the absence of wild-type enzyme.

Substitution of Ly8393 by a serine reduced $k_{cat}$ 600-fold in the direction of degradation and decreased the apparent affinity for P, by a factor of about 5 in the absence of monovalent cations, whereas the $K_m$ value for maltotetraose remained essentially unchanged (Table I). In the direction of synthesis the $k_{cat}$ value was reduced by approximately the same amount, and the apparent binding of Glc-1-P was less affected.

To probe the catalytic role of the ε-amino group of Ly8393, different ligands were tested for their ability to reestablish catalytic activity of the Ly8393-Ser mutant. Amines such as methyl-, ethyl-, and propylamine, ethanalamine, hydroxylamine, and hydrazine neither stimulated nor inhibited the phosphorylase reaction. Only with ammonium ions was a significant 8-fold increase of $k_{cat}$ observed. Enzymatic activity, however, was also stimulated by monovalent alkali cations. Under optimal conditions a 10-fold increase of the reaction rate was found in both directions on the addition of Li+, still a 3-fold increase on addition of Na+ or K+. The dependence of activity on the concentration of cations followed hyperbolic saturation kinetics (Fig. 2). Although a precise determination of the $K_{app}$ values was difficult due to the high concentrations of cations required, the $K_{app}$ for Li+ was estimated to be about 80 mM, the $K_{app}$ values for NH4+, Na+, and K+ were estimated to be 90, 120, and 180 mM, respectively. These values correlate with the ion radii of the cations, with the exception of NH4+. Since the ion radii of K+ is similar to that of NH4+, ammonia ions should show a $K_{app}$ similar to that of K+, which is not the case. The differential behavior of NH4+ is probably due to the different electron shell of the ammonium molecule, which may allow hydrogen bonding to the protein. The saturation kinetic is in contrast to the stimulation of the aspartate aminotransferase Ly8393-Ala mutant by primary amines, where no saturation by high concentrations of primary amines was found (18). The proposal of a specific binding site for monovalent cations received support from the observation that different monovalent cations showed mutual competition. The activity of the Ly8393-Ser mutant in the presence of 100 mM KCl increased upon addi-

**TABLE I**

| Enzyme | $k_{cat}^{s+}$ | $K_m$ | $k_{cat}^{s+}$ | $K_m$ |
|--------|--------------|------|--------------|------|
| Wild-type | 26 | 0.45 | 0.40 | 39 | 1.0 |
| Lys8393-Ser' | 0.045 | 2.3 | 0.61 | 0.06 | 1.23 |
| Lys8393-Gln | 0.06 | 0.43 | 0.40 | 0.081 | 1.0 |

*Activity in the direction of degradation was measured in a coupled assay (see “Material and Methods”).

*Synthesis was measured as release of phosphate from Glc-1-P.

*Activity determined in absence of monovalent alkali cations.

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2. D. Palm, S. Becker, and R. Schinzel, manuscript in preparation.
The failure of primary amines to stimulate activity in the Lys$^{353}$-Ser mutant makes a function of Lys$^{353}$ as a proton donor unlikely. This is in contrast to the chemical rescue of an inactive mutant of aspartate aminotransferase by exogenously added amines, which act as proton transfer catalysts (18). Furthermore, the absence of changes in the pH dependence of the Lys$^{353}$-Ser mutant compared with wild-type supports the conclusion stated above (19).

The distinct properties of the Lys$^{353}$-Ser and the Lys$^{353}$-Gln mutants allow to dissect the role of Lys$^{353}$ into at least two components, a conformational one, important for binding of the substrate phosphate, and a catalytic one, based on the positive charge of the ε-amino group. The pronounced decrease of $k_{cat}$ resulting from the removal of the positive charge of Lys$^{353}$ points to participation of this residue in two key features of phosphorylase catalysis, the requirement of at least a balanced cofactor phosphate monoanion-dianion mixture in the course of the reaction and a close contact between the cofactor phosphate and the substrate phosphate. One function of the positive charge could be to contribute to the balance of charges in the active site. Loss of one positive charge would force the cofactor phosphate to a monoanion state to preserve electrostatic neutrality. Therefore, the $k_{cat}$ for the mutant should be considerably reduced. Monovalent cations, which fit into the cavity created by the Lys$^{353}$-Ser exchange, allowed for partial restoration of the electrostatic environment, but at the expense of reduced substrate affinity.

An indirect participation of the Lys$^{353}$ residue in promoting the close phosphate-phosphate contact can be considered. The positively charged Lys$^{353}$ might be important to align the phosphate of pyridoxal-P by electrostatic interactions. Removal of the salt bridge would allow more flexibility for the phosphate of pyridoxal-P, which in turn renders the orientation of the phosphate-phosphate contact at random and, thereby, affects catalytic efficiency. Addition of cations counteracts the flexibility caused by the mutation, thereby partially restoring activity. Extending earlier observations (12), a delicately balanced rather tight system of residues around the cofactor binding site became apparent, which constitutes one of the prerequisites for the unique function of pyridoxal-P in phosphorylase catalysis.

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