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The phosphate recognition site of *Escherichia coli* maltodextrin phosphorylase

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The role of two positively charged amino acid residues located at the active site of *Escherichia coli* maltodextrin phosphorylase was investigated by site-directed mutagenesis. Substitution of Lys^{539} by an arginine caused a 600-fold reduction, substitution of Arg^{534} by a glutamine caused an even larger 7000-fold reduction of the catalytic rate while substrate binding remained essentially unaffected. Since the $Arg^{534} \rightarrow Gln$ exchange reduces the catalytic rate near to inactivity and even the conservative $Lys^{534} \rightarrow Arg$ exchange caused a marked decrease of activity, the central functional role of both positively charged residues in phosphorylase catalysis anticipated by the crystallographic analysis of the corresponding amino acid residues Arg^{569} and Lys^{574} in the catalytic site of phosphorylase *b* was confirmed.

Structure–function, Site-directed mutagenesis, Steady-state kinetics

1. INTRODUCTION

Pyridoxal 5′-phosphate dependent α-glucan phosphorylases (EC 2.4.1.1) catalyze the first step in the mobilization of storage polysaccharides resulting in the energy-conserving release of glucose 1-phosphate.

Kinetic studies with pyridoxal 5′-*P* analogs and a new class of 'glycosylic' substrate analogs as well as ^{31}P-NMR spectroscopy revealed an unconventional participation of pyridoxal-*P* in phosphorolysis depending on a close contact between the co-factor phosphate and the substrate phosphate (for recent reviews see [1,2]). This mechanism requires highly co-ordinated, complex interactions between co-factor, substrates and enzyme protein and the exclusion of water from the active site to allow non-hydrolytic cleavage in an aqueous environment.

The structural basis for understanding the catalytic mechanism was provided by X-ray studies of the phosphorylase *b*-heptulose 2-*P* complex [3,4] which displays the arrangement of interacting groups when the reaction proceeds in the direction of 'glycogen degradation'. Based on the 3-D structure a critical role of a cluster of amino acids lining the catalytic site proximal to the glucopyranosyl ring and the 'substrate' phosphate (Fig. 1), was anticipated. The high sequence homology (92–100%) of domains involved in substrate binding and catalysis among all glycogen phosphorylases [5] allowed us to probe the function of the corresponding Lys^{539} and Arg^{534} in maltodextrin phosphorylase (Lys^{574} and Arg^{569} in the rabbit muscle sequence) by site-directed mutagenesis. The *Escherichia coli* maltodextrin phosphorylase is especially well-suited for these experiments since, unlike mammalian phosphorylase, it is an unregulated enzyme.

While the functional contribution of Glu^{637}, Tyr^{538} (Glu^{672} and Tyr^{573} in rabbit muscle sequence) and one of the positively charged residues, Lys^{533} (Lys^{568}), were described recently [6,7], the present paper gives a first insight into the functional role of the other two positively charged amino acid side chains at the active site, Arg^{534} and Lys^{539}

2. MATERIALS AND METHODS

2.1. *Strains and plasmids*

E. coli strain pop2158 was obtained from Dr M. Schwartz, Institut Pasteur, Paris. Plasmid pMAP101 was constructed from plasmid pOM13 as described before [8,9]. DNA was manipulated by standard procedures [10]. Restriction endonucleases, T4 DNA-ligase and polynucleotide kinase were used as recommended by the manufacturer (Boehringer Mannheim). Oligonucleotides were prepared on an Applied Biosystem 380A DNA synthesizer and purified by preparative electrophoresis on a denaturating 20% polyacrylamide gel.

2.2. *Mutagenesis*

A 600 bp *Bgl*II-*Pst*I fragment from the *mal*P gene was subcloned into the *Bam*HI and *Pst*I site of M13mp19. Site-directed mutagenesis was accomplished by the procedure of Kunkel [11] with a 24 bp oligonucleotide "CAGATCAAACAGCTGCATGAGTAC in which the CGT codon for Arg^{534} was replaced by a CAG codon for Gln. This exchange, in addition, created a *Pvu*II recognition site to facilitate screening. A *Bst*XI-*Pst*I fragment containing the mutation was recloned into plasmid pMAP101. Accordingly the substitution of

Abbreviations: Pyridoxal-*P*, pyridoxal 5′-phosphate; glucose-1-*P*, α-D-glucopyranose-1-phosphate

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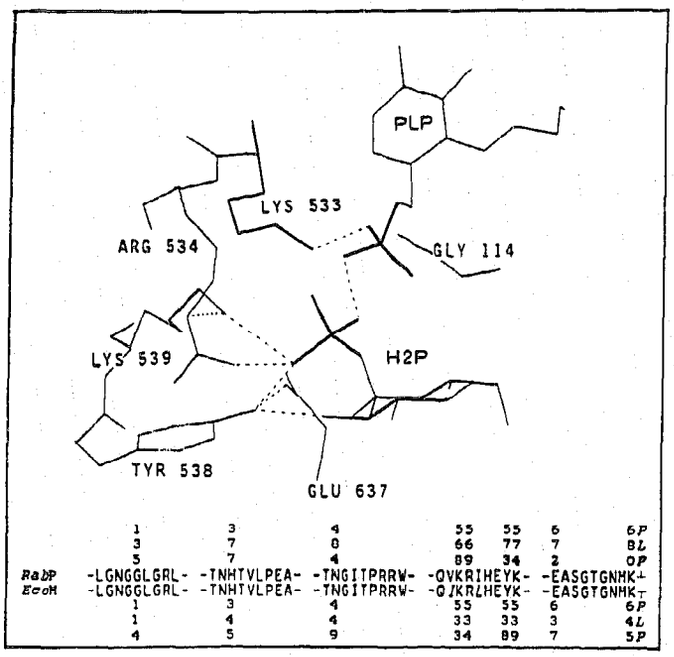

	1	3	4	55	55	6		6P
	3	7	0	66	77	7		8L
	5	7	4	89	34	2		0P
RabP	-LGNGGLGRL-	-TNHTVLPEA-	-TNGITPRRW-	-QVKRIHEYK-		-EASGTGNMK-		
EcoH	-LGNGGLGRL-	-TNHTVLPEA-	-TNGITPRRW-	-QIKRLHEYK-		-EASGTGNMK-		
	1	3	4	55	55	6		6P
	1	4	4	33	33	3		4L
	4	5	9	34	89	7		5P

Fig. 1. The proposed active site structure of maltodextrin phosphorylase based on the structure of the rabbit muscle phosphorylase (adapted from [3]). Inset: Alignment of the substituted amino acid residues of phosphorylase *b* (RabP) to *E. coli* maltodextrin phosphorylase (EcoM). HP = heptulose 2-phosphate.

Lys^{539} by an arginine was introduced using a 23 bp oligonucleotide "GCATGAGTACCGGCGCCAGCACC" in which the AAA codon for Lys^{539} was altered to a CGG codon, coding for Gln and creating a *Nar*I recognition site. The nucleotide exchanges were confirmed by sequencing.

2.3. *Enzyme purification*

Wild-type and mutant enzyme were purified as described [6,12]. A chromatography on a FPLC Mono-Q anion-exchange column, was introduced as an additional purification step. The enzyme was eluted from the Mono-Q column previously equilibrated with 10 mM Tris-acetate, 1 mM EDTA, pH 6.9 by a linear gradient (0–0.3 M NaCl in 10 mM Tris-acetate, 1 mM EDTA, pH 6.9) and dialysed against 10 mM Tris-acetate, 1 mM EDTA, 50 mM KCl, pH 6.9.

Purification of low-activity mutant proteins was followed by SDS-PAGE. The pyridoxal-*P* content was determined as described by Wada and Snell [13]. Protein was measured by the method of Bradford [14] or from the absorbance at 280 nm, using $E^{0.1\%}_{1cm} = 1.36$.

2.4. *Maltodextrin phosphorylase assays*

Enzyme activity was assayed at 30°C in the direction of phosphorolysis in a coupled assay as described [6]. Arsenolysis and hydrolysis were measured by determining the release of glucose by a spectrophotometric assay using glucose dehydrogenase [6]. In the direction of synthesis the enzyme was assayed by measuring the release of P_i [6,15]. Initial velocity studies were performed at 30°C by varying the concentration of the substrates within a 10-fold range. The kinetic parameters were calculated by non-linear regression using the GRAFIT program [16]. Changes in free energy were calculated by using $G^{\circ\prime} = -RT \ln(k_{cat\,mut}/K_{m\,mut}/(k_{cat\,wt}/K_{m\,wt})$ [17].

3. RESULTS AND DISCUSSION

Site-directed mutations were introduced into the cloned *malP* gene of *E. coli* [9] by the uracil template method [11] with an efficiency of more than 60%. The mutant enzymes were overexpressed and purified in high yield as described [6] with the exception of the $Lys^{539} \rightarrow$Ser substitution, for which no expression of the mutated protein was observed [18]. All mutant enzymes were purified to electrophoretic homogeneity. UV-spectra of the mutant enzymes were identical to the spectrum of the wild-type enzyme, in particular the $OD_{330/280}$ ratio was left unchanged, indicating that these mutations had no influence on the quantity of pyridoxal-*P* binding. The co-factor content was determined for both mutants to be 1 mol pyridoxal-*P*/mol protein. The heat inactivation curves at 60°C displayed no difference in stability compared to the wild-type enzyme for both mutant enzymes. Hence we conclude that no essential structural changes had been introduced by the substitutions described.

The steady-state parameters for wild-type and mutant enzymes were determined at fixed concentrations of the second substrate as described in section 2 and are listed in Table I. The $Arg^{534} \rightarrow$Gln exchange caused a drastical reduction of the k_{cat} value by a factor of at least 7000 both in the direction of synthesis and degradation of maltodextrin, while the apparent binding of all physiological substrates seemed to be only slightly affected. This points to a marked decrease of binding in the transition state [19] and would correspond to a loss in transition state binding energy of about 5 kcal. However, since the measured activities were very low and the K_m values remained unaffected, a contamination by traces of wild-type enzyme cannot be totally excluded in the case of the $Arg^{534} \rightarrow$Gln mutant enzyme. On the other hand, the K_i value of the arsenate ions, which are competing with P_i for the phosphate binding site, increased significantly from 0.5 mM for the wild-type to 2.7 mM determined for the $Arg^{534} \rightarrow$Gln mutant (Table I). This fact rules out a significant contamination by the wild-type enzyme.

The catalytic activity of the $Lys^{539} \rightarrow$Arg substituted

Table I

Steady-state kinetic parameters of the $Lys^{539} \rightarrow$Arg and the $Arg^{534} \rightarrow$Gln substituted maltodextrin phosphorylase

	Wild-type[c]	Arg^{534}-Gln	Lys^{539}-Arg
Phosphorolysis			
K_m (mM) P_i	0.5	0.4	1.1
K_m (mM) Glc_7	0.5	0.3	0.4
k_{cat} (s^{-1})	28	0.004	0.046
Glycogen synthesis			
K_m (mM) Glc-1-P	1.1	0.4	0.4
k_{cat} (s^{-1})	36	0.008	0.015
Hydrolysis[a]			
k_{cat} (s^{-1})	0.0032	0.0002[b]	0.0002[b]
K_i (AsO_4^{2-})	0.54	2.7	6.5

[a] Measured as release of glucose from Glc_7
[b] Values differ by a factor of 2 from batch to batch
[c] Data from [6]

enzyme was markedly reduced (600-fold), but not to the extent observed for the Arg^{534}→Gln mutant. Substrate affinities in the ground state, measured as the apparent binding, for all substrates were only slightly affected by the Lys^{539}→Arg exchange, with the exception of a small but significant increase of apparent binding of inorganic phosphate. Since the present mutation preserves the positive charge and can be, despite the increase of side-chain volume, considered a conservative replacement, the order of magnitude of the rate effect is noteworthy. However, while a replacement of Lys^{539} by an arginine allowed the isolation of stable, but rather inactive enzyme, replacement of the same lysine residue by a small and uncharged residue like serine led to a case where no stable enzyme protein could be isolated. From these observations it may be concluded that ligand binding in the ground state and protein stability tolerate changes in the size of the amino acid side chain within narrow limits, while catalysis requires a more precise co-ordination of the active site residues. Similar effects were observed as a consequence of the Glu^{637}→Asp exchange, where minor changes in K_m were found despite a large reduction of k_{cat} [6]. Since even moderate changes like in the present case caused such severe effects on maltodextrin phosphorylases, a central role of Lys^{539} in catalysis is strongly indicated. The rather small effects of the Lys^{539}→Arg mutant on binding are not unexpected, since the positive charge of the side chain should account for the major contribution to substrate binding in the ground state.

In the case of the Arg^{534}→Gln mutant enzyme, too, the $K_{i,As}$ value increased or accordingly the apparent affinity for the inhibitor arsenate was decreased markedly (10-fold). This might be due to the different volume of the arsenate anion compared with the phosphate anion. In contrast to the phosphate ion binding of the larger arsenate ion is weakened in response to the small structural changes introduced by the mutation.

For the Lys^{539}→Arg mutant a shift of the pH-optimum to a more basic value (from pH 6.5 to pH 7.5) was observed. The data illustrate (Fig. 2) that the basic branch was more affected than the acidic branch of the reaction. This indicates at least a partial contribution of Lys^{539} to the more basic pK value of the reaction.

Similar to other active site mutants of maltodextrin phosphorylase [6] the Arg^{534}→Gln mutant exhibits a relatively high 'error rate', the release of glucose instead of Glc-1-*P* from oligosaccharides. The hydrolysis/phosphorolysis ratio, which is 1/9000 or less for the wild-type enzyme, was found to have increased to approximately 1/20 for the Arg^{534}→Gln mutant. Although, due to the low activities, an accurate value was difficult to obtain, the error ratio appeared to be higher than the ratios observed for other active site mutants (Table I). In contrast, the relative error ratio (1/230) of the Lys^{539}→Arg mutant appeared to be lower than those of other active site mutants of

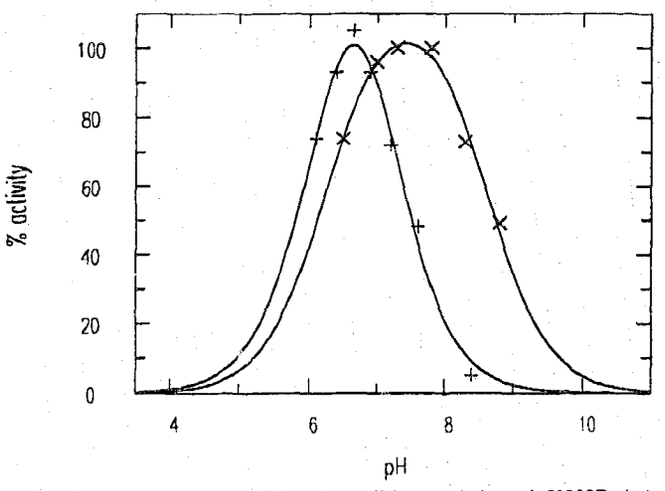

Fig. 2. pH dependence of k_{cat} for wild-type (+) and K539R (×) substituted maltodextrin phosphorylase.

maltodextrin phosphorylase investigated [6]. This observation can be interpreted as the consequence of the conservative lysine to arginine substitution which obviously does not allow for additional binding of water at the active site to the extent observed for the other mutant enzymes, therefore relatively reducing 'hydrolytic competition'.

The proposed central role of the two positive charged amino acids Arg^{534} and Lys^{539} as anticipated from the structure of the phosphorylase *b*–heptulose 2*P* complex [3,4] and from chemical modification studies of the corresponding Arg^{569} in phosphorylase *b* [20,21] was confirmed by this site-directed mutagenesis study showing the essential contribution to phosphate recognition. The almost complete loss of activity observed for the isosteric Arg^{534}→Gln mutant enzyme and the still pronounced reduction observed for the Lys^{534}→Arg mutant showed that both positive charges are required for a fully active enzyme. Quite surprisingly the substrate binding capabilities of the Arg^{534}→Gln mutant seemed to be mainly unaffected. Since the incoming phosphate is bound even below the pH optimum of the phosphorylase reaction, most probably in the monoanionic state, we assume that a single positive charge is sufficient to support binding, therefore explaining why the Arg^{534}→Gln substitution does not affect the ground state binding of phosphate.

While Lys^{533}, the third positively charged residue identified at the active site, is involved in positioning the phosphate group of the cofactor [7], Lys^{539} might be involved in binding of the substrate phosphate both in the ground and transition state most probably through electrostatic interactions. This leaves an important role to Arg^{534} in maintaining the balance of charges at the active site and in aligning the cofactor phosphate and the substrate phosphate allowing the proton transfer mechanism postulated [2]. Arg^{534} seems to be a substantial part of the phosphate–phosphate contact

subsite of the active site similar to the role of Glu^{637} in forming the phosphate–carbohydrate contact region [6]. To clarify the detailed mechanistical role of these two residues further, kinetic studies with substrate analogs and by ^{31}P-NMR studies will be performed.

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