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Identification of an allosteric site residue of a fructose 1,6-bisphosphate-dependent L-lactate dehydrogenase of *Thermus caldophilus* GK24: production of a non-allosteric form by protein engineering

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Fructose 1,6-bisphosphate (Fru-1,6-P_2)-dependent L-lactate dehydrogenase (LDH) of *Thermus caldophilus* GK24 can be converted to a Fru-1,6-P_2-independent form on modification with Arg-specific reagents. After trypsin digestion of the modified LDH, a peptide containing a modified Arg residue was purified and sequenced, and the modified Arg residue was identified as Arg-173. Subsequently, Arg-173 was replaced with Gln to remove the positive charge by site-directed mutagenesis. The mutant LDH was independent of Fru-1,6-P_2, like non-allosteric vertebrate LDHs. Thus, Arg-173 was concluded to be located in the allosteric site and to be responsible for allosteric regulation of the LDH.

Allosteric L-lactate dehydrogenase; Fructose 1,6-bisphosphate-binding site; Chemical modification; Protein engineering; Site-directed mutagenesis; (*Thermus caldophilus*)

1. INTRODUCTION

The structures and functions of L-lactate dehydrogenases (LDHs) (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) from muscle [1-5], heart [1,5,6] and testis [1,7,8] have been studied in great detail both biochemically and crystallographically. The amino acid sequences of the allosteric LDHs of *Thermus caldophilus* GK24 [9], *Lactobacillus casei* [10], *Bacillus stearothermophilus* [11] and *B. subtilis* [12] show high homology with each other and with those of non-allosteric vertebrate LDHs (about 40% identity). A crystallographic study demonstrated the high structural similarity between LDHs of *L. casei* and dogfish muscle [13], suggesting that bacterial and vertebrate LDHs have similar three-dimensional structures.

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Abbreviations: LDH, L-lactate dehydrogenase; Fru-1,6-P_2, fructose 1,6-bisphosphate

The activity of *T. caldophilus* LDH is dependent on fructose 1,6-bisphosphate (Fru-1,6-P_2) [14,15]. We studied the mechanism underlying allosteric regulation of the LDH by means of ^1H NMR [16] and chemical modification [17]. The LDH can be desensitized to Fru-1,6-P_2 in the activated state through Arg-specific chemical modification in the presence of NADH and oxamate, and the absence of Fru-1,6-P_2 [17]. In the present study we found that Arg-173 of the LDH was modified by Arg-specific reagents, and that a mutant LDH, which was obtained by replacing Arg-173 with Gln by site-directed mutagenesis, was independent of Fru-1,6-P_2, like non-allosteric vertebrate LDHs.

2. MATERIALS AND METHODS

2.1. *Isolation of a peptide containing a modified Arg residue*

The *T. caldophilus* LDH was purified as described previously [14]. 5 mg of the enzyme was dissolved in 5 ml of 50 mM sodium borate buffer (pH 7.5) containing 10 mM NADH and 50 mM oxamate in the absence and presence of 10 mM Fru-1,6-P_2, and then 2,3-butanedione was added at 2.5 mM, as described previously [17]. The modification reaction was car-

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ried out at 30°C for 30 min. After dialysis against 5% acetic acid and lyophilization, the modified LDH was denatured in 50 mM sodium borate buffer (pH 8.0) containing 10 M urea at 37°C for 10 min. The denatured enzyme was digested with trypsin and the resultant tryptic peptides were isolated by HPLC as described previously [9]. The peptide fragment containing a modified Arg residue was collected. After evaporation, the peptide was dissolved in 0.1 M ammonium acetate, followed by incubation at room temperature for 24 h to regenerate the Arg residue [18,19]. On the second HPLC, the peptide appeared as a single peak at a different position from in the first chromatography. The purified peptide was sequenced as described previously [9].

2.2. *Oligonucleotide-directed site-specific mutagenesis*

A mutant LDH with Gln-173 was constructed through mutation of the Arg-173 codon [9], CGG, to CAG, using a synthetic 21-base oligodeoxyribonucleotide, 5'-GCCCGCTTCCAGGCCCTTCTG-3', as a primer to generate the mutant LDH gene in a plasmid vector as described previously [20]. The entire LDH gene was sequenced, as described previously [9], to prove that only the Gln-173 mutation had occurred. The wild-type and mutant LDH genes were inserted into the pUC vector and then expressed in *Escherichia coli* through the *lac* promoter, as described previously [9].

2.3. *Enzyme assay*

The enzymes were purified from cells of *E. coli* JM83 [*ara* Δ(*lac-proAB*) *thi rpsL* φ80Δ*lacZ*ΔM15], carrying the plasmids containing the wild-type and mutant LDH genes, by affinity chromatography on oxamate-Sepharose 4B [14]. LDH activity was assayed at 50°C by the method described previously [14].

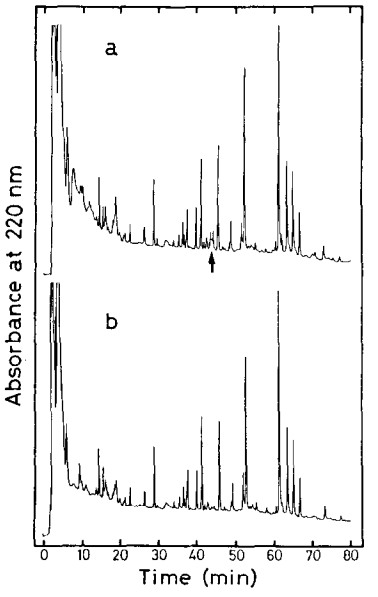

Fig.1. Isolation of a peptide containing a modified Arg residue from the trypsin-digested 2,3-butanedione-treated *T. caldophilus* LDH by HPLC. The peptide fragment containing the modified Arg residue was identified by comparing the elution profiles of the peptides obtained for the LDHs modified in the absence (a) and presence (b) of 10 mM Fru-1,6-P_2. The peptide fraction which was observed in (a) but not in (b) (indicated by an arrow) was collected.

3. RESULTS AND DISCUSSION

Fru-1,6-P_2-dependent *T. caldophilus* LDH can be converted to a Fru-1,6-P_2-independent form on modification with Arg-specific reagents (2,3-butanedione and 1,2-cyclohexanedione) [17]. We attempted to determine the position of the Arg residue responsible for the desensitization of the enzyme. Fig.1 shows high-performance liquid chromatograms of tryptic peptides of the LDH modified with 2,3-butanedione in the absence (fig.1a) and presence (fig.1b) of Fru-1,6-P_2. The peptide fraction in fig.1a (indicated by an arrow) was collected: this should contain the peptide which was protected from trypsin digestion by the Arg modification. After regenerating the Arg residue and purifying the peptide, the amino acid sequence was determined to be Phe-Arg*-Ala-Leu-Leu-Ala-. The tryptic peptide likewise purified from the 1,2-cyclohexanedione-modified LDH was sequenced and found to be Phe-Arg*-Ala-Leu-Leu-Ala-Glu-His-Leu-Arg. Arg* was thus found to be the amino acid residue which had been modified by the reagents. By comparing the sequences with the whole primary structure [9], Arg* was determined to be Arg-173 in the LDH.

In the modification reaction, Fru-1,6-P_2 inhibited the modification of the Arg-173 residue (fig.1). This suggests that Fru-1,6-P_2 interacts directly with Arg-173. On allosteric activation by Fru-1,6-P_2, the direct interaction between Fru-1,6-P_2 and Arg-173 probably leads to activation of the LDH. On both chemical modification and allosteric activation, the positive charge of the guanidino group of Arg-173 seems to be lost. Thus, we examined the mutational effect of Arg-173 on the allostericity of the LDH by means of site-directed mutagenesis, replacing Arg-173 with Gln to remove the positive charge, the bulk and hydrophilicity being retained.

The replacement of Arg-173 with Gln caused a drastic change in the allostericity of the LDH: the resultant mutant LDH was no longer an allosteric enzyme, its activity being independent of

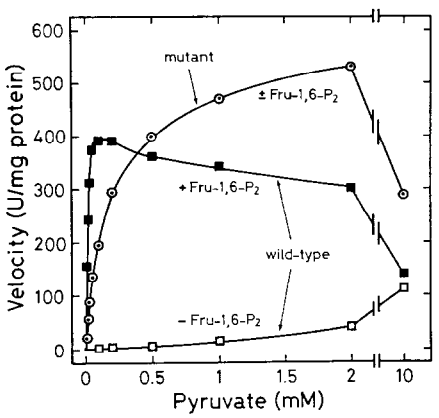

Fig.2. Pyruvate saturation curves for the wild-type (Arg-173) and mutant (Gln-173) LDHs of *T. caldophilus*. The enzymes were purified from cells of *E. coli* JM83 carrying the plasmids containing the wild-type and mutant LDH genes. LDH activity was assayed at 50°C by the method described previously [14]. 10 μl of an enzyme sample (0.28 and 0.37 μg of the wild-type and mutant LDHs, respectively) was added to a 500 μl assay mixture containing sodium 4-morpholine propanesulfonate (Mops) buffer (50 mM, pH 7.1), NADH (0.2 mM), sodium pyruvate (as indicated), and 0 mM (open symbols) or 0.2 mM (closed symbols) Fru-1,6-P_2. Squares and circles denote the activities of the wild-type and mutant LDHs, respectively. One unit (U) was taken as the amount of the enzyme required to catalyze the oxidation of 1 μmol NADH/min under the above conditions.

(0.15 mM) and NADH (0.3 mM, not shown), and V (530 U per mg of protein) of the mutant LDH did not differ in the absence and presence of Fru-1,6-P_2. The V of the mutant LDH was almost identical to that for wild-type LDH (550 U per mg of protein, calculated from the data in fig.2), when assayed with the effector. These results indicate that Arg-173 is located in the allosteric site of the LDH and that the positive charge of Arg-173 plays a decisive role in its allosteric regulation.

It is noteworthy that the K_m values of the mutant LDH were about ten times greater than those of the wild-type enzyme, when assayed with the effector, i.e., 0.026 mM for pyruvate (calculated from the data in fig.2) and 0.03 mM for NADH (not shown), indicating that the local conformation of the active site of the mutant LDH is not identical to that of the wild-type enzyme, when bound to Fru-1,6-P_2. Some residue(s) other than Arg-173 may also be involved in the allosteric regulation of the LDH.

X-ray crystallography allowed identification of the amino acid residues forming the anion-binding site of vertebrate LDHs [2,21]. His-188 of the *L. casei* LDH, located in the allosteric site, is homologous to His-188, which is part of the anion-binding site, of vertebrate LDHs [21]. Arg-173 involved in the allosteric regulation of the *T. caldophilus* LDH is also located in a region corresponding to the anion-binding site of vertebrate LDHs [2,21]. Arg-173 as well as His-188 is conserved in all of the LDHs so far studied [5,8–12]. These findings strongly suggest that the allosteric site of allosteric LDHs corresponds to the anion-binding site of vertebrate LDHs, as described previously [21].

The anion-binding site is situated close to the molecular P-axis in one of the three subunit interfaces [2]. In the tetrameric molecule, two each of the four anion-binding sites face one another, at close distance. Holbrook and his colleagues [22,23] studied an allosteric LDH of *B. stearothermophilus* and showed that its Arg-173 residue regulates the state of quaternary structure (the assembly of two dimers) through a charge-repulsion mechanism. Our findings support theirs, and the allosteric regulation of *T. caldophilus* LDH is probably similar to that of *B. stearothermophilus* LDH.

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