Subunit-hybrid enzymes of mutant tetrameric L-lactate dehydrogenases from *Bifidobacterium longum* were studied in an examination of the mechanism of allosteric activation by fructose 1,6-bisphosphate. We earlier developed an *in vivo* method for subunit hybridization in *Escherichia coli* and the hybrids formed were a mixture with different subunit compositions. The *B. longum* hybrids were separated by anion-exchange chromatography with a mutational tag. Hybrids formed between fructose 1,6-bisphosphate-desensitized subunits and wild-type subunits and also between fructose 1,6-bisphosphate-desensitized subunits and catalytically inactive subunits. Kinetic analyses of the hybrid enzymes showed that (i) those residues from two symmetrically related subunits that constituted the fructose 1,6-bisphosphate-binding site could bind fructose 1,6-bisphosphate and activate the enzyme only if intact, (ii) hybrids with only one functional fructose 1,6-bisphosphate-binding site were fully sensitive to fructose 1,6-bisphosphate, but the allosteric equilibrium had shifted partially, and (iii) activation by fructose 1,6-bisphosphate at the fructose 1,6-bisphosphate-binding site was transmitted to the active sites through a quaternary structural change, not through direct conformational change within a subunit. These results are evidence of the validity of the concerted allosteric model of this enzyme based on *P* and *R*-state structures in the same crystal lattice proposed earlier.

*L*-Lactate dehydrogenases (LDHs)† (EC 1.1.1.27) from some bacteria are allosteric enzymes with sigmoidal kinetics for pyruvate (homotropic activation), and are allosterically activated by fructose 1,6-bisphosphate (FBP) (heterotropic activation) (1), unlike non-allosteric vertebrate LDHs (2). All of these enzymes are tetramers and composed of identical subunits with molecular masses of 30–35 kDa.

*Bifidobacterium longum* LDH (BLLDH) is an FBP-dependent allosteric LDH (3, 4), and its crystal structures have been reported (5, 6). Four subunits of BLLDH are related by three molecular 2-fold axes named *P*, *Q*, and *R* (Fig. 1) as in the definition of vertebrate LDHs (7). BLLDH has subunit contacts only in the subunit interfaces along the *P* and *Q*-axes, but not in that of the *R*-axis. The tetramer has four active sites but only two FBP-binding sites. The FBP-binding site is at the *P*-axis interface and one site is composed of residues from two subunits related by the *P*-axis, where the anion binding site of vertebrate LDHs is (8). Arg-173² and His-188 of this site form salt bridges with phosphate moieties of the bound FBP molecule. The active site is at the *Q*-axis subunit interface, in which His-195 is essential for enzyme catalysis and substrate binding (2).

In the crystal structure of BLLDH reported more recently (6), two kinds of tetramers, one in a no-affinity state (*T*-state) and one in a high-affinity state (*R*-state) to the substrate, were found in the same crystal lattice, and a model of allosteric transition based on the concerted model by Monod et al. (9) was proposed. This “Monod-Wyman-Changeux crystal” has not only provided evidence for the existence of two different allosteric states within this protein, but also allowed detailed comparison of the states. The activation process of the enzyme, however, including communication between the active site and the effector site, remains unclear since crystallography gives only static images of the two extreme states.

To characterize the activation of the enzyme, we applied the subunit hybridization technique to BLLDH. Previously, we reported an *in vivo* hybridization technique that used two-plasmid transformants of *Escherichia coli* (10). We did produce subunit hybrids in this way, but transformants were unstable because two plasmids with different antibiotic resistance were harbored in the cells. Moreover, the mutational tag designed for separation of the hybrids was unsuitable and affected the allostericity of the enzyme. Therefore, interpretation of the results was difficult. Here, we developed a new *in vivo* subunit hybridization system with a plasmid that carries two BLLDH genes tandemly. This system is more stable than the previous one. The mutational tag was redesigned so as not to affect the allosteric properties of the enzyme.

Here we describe the production and separation of subunit hybrids with this system. One group of hybrids were between FBP-desensitized and wild-type subunits. The other group were between FBP-desensitized and inactive subunits. From the kinetics of the hybrids obtained, we found how activation at the FBP binding site is transmitted to the active sites. These results are consistent with the crystal structure-based allosteric model of the enzyme (6).

**MATERIALS AND METHODS**

**Chemicals—**Sodium pyruvate and NADH were purchased from Boehringer Mannheim and Life Technologies Oriental (Tokyo, Japan), respectively. The enzymes used for DNA manipulation were obtained from Boehringer Mannheim, Takara Shuzo (Kyoto, Japan), and Toyobo.

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† The abbreviations used are: LDH, L-lactate dehydrogenase; FBP, fructose 1,6-bisphosphate; BLLDH, *B. longum* L-lactate dehydrogenase; native PAGE, nondenaturating polyacrylamide gel electrophoresis.

* Residue numbers are given in the N-system (32).
Allosteric Activation of Hybrid L-Lactate Dehydrogenase

Biochemicals (Tokyo, Japan).

Mutagenesis—Oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems model 391). Site-directed mutagenesis was done with a Mutagene in vitro mutagenesis kit (Bio-Rad) as described by Kunkel (11), and the DNA fragments were sequenced to check for mutations. Oligonucleotides 5'-CTC GCC GCT TCT GAG GAC TCT GCC GAG AGC-3' and 5'-ATC GCC GCT TCT GAG AAC GCC GAC GCC TCA GGA GTG-5' were synthesized to obtain mutant enzymes K316E/R317D and H195N, respectively. From the expression vector for the K316E/R317D mutant derived from pUBM9 (12), a 350-base pair SalI-Sall fragment that contained the region for His-195 was replaced with a SalI-Sall fragment of Asn-195 to construct a H195N/K316E/R317D mutant. The FBP-desensitized mutant with R173Q/H188Y was described previously (10).

Construction of Expression Vectors for in Vivo Hybridization—Expression vectors for in vitro hybridization were constructed to carry two BLLDH genes tandemly. Both contained the tac promoter and a transcriptional terminator. Expression vectors for mutant enzymes were prepared from pUBM9 (12) by site-directed mutagenesis as described above. In these plasmids, a BamHI unique site is found before the promoter and a PvuII unique site is found after the terminator. A 1.6-kilobase BamHI-PvuII fragment was used as a cassette of the complete BLLDH gene. Expression vectors were constructed for hybrid formation between desensitized and wild-type subunits and for hybrid formation between desensitized and inactive subunits. For the first, the BamHI-PvuII fragment of the desensitized LDH gene was blunted and inserted into the PvuII site of the expression vector of wild-type LDH. For the second, the DNA fragment of an inactive LDH gene was similarly inserted into the PvuII site of the expression vector of desensitized LDH. All mutant enzymes used had Cys-210B replaced by serine.

One unit was defined as the amount of enzyme that converted 1 mol of substrate/min. Michaelis equation.

\[ v = \frac{V_{\text{max}} (\text{pyruvate})^n}{(\text{pyruvate})^n + (S_b)^n} \] (Eq. 1)

Hybrid D113, with only one active site, was analyzed with the Michaelis equation.

Mutant Enzymes—Together with the wild-type BLLDH (abbreviated W), we used two kinds of mutants in which the FBP-binding or active site was impaired. Mutant enzyme D, which is desensitized to FBP, was obtained by replacement of Arg-173 and His-188 with glutamine and tyrosine, respectively (Fig. 1). Hybrid enzyme I, which had no catalytic activity, was obtained by replacement of His-195 with asparagine. The specific activity of enzyme I was 0.04 units/mg of purified protein, negligible compared with that of the wild-type enzyme (~1000 units/mg). Results of measurement of NADH fluorescence (16) showed that this inactive enzyme could bind the coenzyme NADH. CD spectra of all mutants were almost the same (data not shown).

Design of Subunit Hybrids—We studied two groups of subunit hybrid enzymes. First, we constructed D-W hybrids, between D and W subunits, to characterize the "odd" FBP-binding site composed of residues from the W and D subunits (Fig. 2c). Next, D-I hybrids, between D and I subunits, were constructed for use in an examination of allosteric communication between the FBP-binding and active sites. In other words, this study was undertaken to find whether the activation occurs within the subunit (intra-subunit activation) or indirectly through a quaternary structural change in the tetramer (inter-subunit activation). The I subunit could form FBP-binding sites but was catalytically inactive, so we could observe the enzyme activity of the D subunit only.
BLLDH occurred in *E. coli* cells and a mixture of hybrids was produced, as described previously (10). To separate these hybrids, we modified the surface charge of one of the pairs of subunits. For a negatively charged tag, Lys-316 and Arg-317 were replaced with glutamic acid and aspartic acid, respectively. These residues are in the last helix, sticking out into the solvent and far from both the active site and the FBP-binding site (Fig. 1). Allosteric properties of the charge-tagged enzyme were not affected by these mutations (see Fig. 3c), but the expression level in *E. coli* cells was reduced to about 50% that of the untagged enzyme, for an unknown reason. The negatively charged tag could produce a difference in the total surface charge of different hybrid tetramers. This difference made separation of these hybrids possible by anion-exchange chromatography (Fig. 2a) or native PAGE (Fig. 2b).

**Production and Separation of Hybrid Enzymes—**Expression vectors for the hybrid enzymes carrying two LDH genes tan-
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The D-W hybrids expressed in E. coli cells gave five bands on native PAGE (Fig. 2b, lanes 0). The band intensities were well simulated by binomial distribution of tetrameric hybrids between D and W subunits with the molar ratio of 2:1 (untagged subunit: charge-tagged subunit). This finding means that random hybridization between D and W subunits had occurred. The five bands at regular intervals arose from five differently charged hybrids. By anion-exchange chromatography, the same D-W hybrids separated, to our surprise, into six peaks (Fig. 2a). Each peak seemed to be of a hybrid with a different composition and structural arrangement of subunits from the others. The results of this chromatography and of native PAGE (Fig. 2b), and the kinetic data described below were referred to when we named the hybrids in peaks 1 to 6 D4, D3W1, D2W2-QR, D2W2-P, D1W3, and W4 (Fig. 2c), respectively. D2W2-QR in peak 3 and D2W2-P in peak 4 had the same mobility on native PAGE. The ratio of the areas of the peaks corresponding to these hybrids in anion-exchange chromatography was about 2:1 (1.7:1 in Fig. 2a). Re-chromatography of these two hybrids from separate peaks gave only peaks at the same positions as before. D2W2-P contained two D subunits along the molecular P-axis, with one intact FBP-binding site. On the other hand, D2W2-QR was a mixture of the two other hybrids with two D subunits along the Q- and R-axes, without an intact FBP-binding site (Fig. 2c).

For D-I hybrids also, native PAGE gave five bands and anion-exchange chromatography gave six peaks (data not shown), like the D-W hybrids. The level of expression of the charge-tagged I subunit was again about 50% that of the D subunit. The hybrids in peaks 1 to 6 were named D4, D3I1, D2I2-QR, D2I2-P, D1I3, and I4, like D-W hybrids.

Kinetic Properties of D-W Hybrids—Pyruvate saturation curves of the D-W hybrids with and without FBP were classified into three types (Fig. 3, a-c). D4, D3W1, and D2W2-QR were desensitized to FBP (Fig. 3a), so they had no intact FBP-binding sites. Their pyruvate saturation curves were sigmoidal, still showing homotropic activation by the substrate. The Hill coefficients of D4, D3W1, and D2W2-QR in the absence of FBP were 2.2, 2.3, and 2.4, respectively. D2W2-P, D1W3, and W4 were activated by FBP. The concentrations of FBP for half-maximal activation, $S_{0.5}$, of D2W2-P, D1W3, and W4 were 13, 7, and 6 nM, respectively; they had practically the same sensitivity to FBP. The greatest difference among them was sigmoidality of the pyruvate saturation curves in the presence of FBP. In the presence of 0.1 mM FBP, the curve for W4 was almost hyperbolic (Fig. 3c) but the curves for D2W2-P and D1W3 were sigmoidal (Fig. 3b). The Hill coefficients of D2W2-P, D1W3, and W4 in the presence of FBP were 1.9, 2.7, and 1.3, respectively. The concentrations of pyruvate for half-maximal activity, $S_{0.5}$, of D2W2-P, D1W3, and W4 were decreased by the addition of FBP and could be calculated with smaller error than without FBP (Fig. 4a). The $S_{0.5}$ of W4 was smaller than the values for D2W2-P and D1W3. The difference seems to arise from D2W2-P and D1W3 having only one intact FBP-binding site but W4 having two. These results agree with predictions from the crystal structure-based concerted allosteric model (discussed below).

The results were different from our previous ones (10). In the previous study, the D2W2 hybrid did not separate into two peaks. The hybrids that contained one or more D subunits were desensitized and had pyruvate saturation curves similar to the curve of D4. The separation tag (two charged residues) added at the C-terminal seems to have caused a structural change in the D subunit, constraining quaternary subunit rotation. In this study, the tag position was chosen so as not to affect the allosteric properties of the enzyme, and accompanying steric effects enabled D2W2 “isomers” to be separated.

Kinetic Properties of D-I Hybrids—Pyruvate saturation curves for D-I hybrids were shown in Fig. 5. D4, D3I1, and D2I2-QR were desensitized to FBP (Fig. 5a), so these hybrids had no intact FBP-binding sites. Their saturation curves for pyruvate were sigmoidal. On the other hand, D2I2-P (Fig. 5b) and D1I3 (Fig. 5c) were activated by FBP. These results suggest that the enzyme activation by FBP was transmitted from the I subunits to the D subunit(s) via the $Q$-axis interface, because a functional FBP-binding site was formed in the $P$-axis interface between the two I subunits, and because the D subunit(s) touches I subunit(s) in the $Q$-axis interface. I4 was always inactive (data not shown). As I subunits had no activity, $V_{max}$ was nearly proportional to the number of intact active sites in the hybrids (Figs. 5 and 6). In Fig. 6, the two upward-deviating points of $V_{max}$ for hybrid enzymes with one and two active subunits are those of D1I3 and D2I2-P, respectively, in the presence of FBP. Hybrids with fewer intact active sites had saturation curves for pyruvate that were less sigmoidal and when curves were less sigmoidal, the Hill coefficient was lower (Fig. 6). D1I3 had hyperbolic saturation curves both with and without FBP (Fig. 5c), and they fit the Michaelis equation well. The $S_{0.5}$ of D2I2-P and D1I3 decreased when FBP was added (Fig. 4b). $A_{0.5}$(FBP) values of D2I2-P and D1I3 at 5 mM pyruvate were 13, 7, and 6 nM, respectively; they had practically the same sensitivity to FBP. The greatest difference among them was sigmoidality of the pyruvate saturation curves in the presence of FBP. In the presence of 0.1 mM FBP, the curve for W4 was almost hyperbolic (Fig. 3c) but the curves for D2W2-P and D1W3 were sigmoidal (Fig. 3b). The Hill coefficients of D2W2-P, D1W3, and W4 in the presence of FBP were 1.9, 2.7, and 1.3, respectively. The concentrations of pyruvate for half-maximal activity, $S_{0.5}$, of D2W2-P, D1W3, and W4 were decreased by the addition of FBP and could be calculated with smaller error than without FBP (Fig. 4a). The $S_{0.5}$ of W4 was smaller than the values for D2W2-P and D1W3. The difference seems to arise from D2W2-P and D1W3 having only one intact FBP-binding site but W4 having two. These results agree with predictions from the crystal structure-based concerted allosteric model (discussed below).
The intrinsic affinities of their FBP-binding sites should be unchanged, but their $A_{0.5}(\text{FBP})$ were larger than that of the wild-type enzyme W4. For D2I2-P and D1I3, the activities with and without FBP were not so different at 5 mM pyruvate (see Fig. 5, b and c) as for W4 (Fig. 3c). These differences in the degree of FBP activation seemed to result in the $A_{0.5}(\text{FBP})$ values of the hybrids being higher than the value of the wild-type enzyme.

**DISCUSSION**

**Peak Assignment**—From the mobility of the hybrid proteins on native PAGE, the numerical subunit compositions of the substance in each peak on Mono-Q chromatography could be assigned. Structural assignment for D2W2-P and D2W2-QR was based on the following reasoning, which also applied to the D-I hybrids D2I2-P and D2I2-QR. A hybrid composed of two D subunits and two W subunits (D2W2) should be a mixture of three isomers that have two D subunits related by the P-, Q-, or R-axis (see Fig. 2c). These three isomers have the same overall charge, so they had exactly the same mobility on native PAGE. Probably for some steric reason, however, these isomers separated into two peaks on anion-exchange chromatography. From the stoichiometry of their peak areas in chromatography, it was seen that two of the three isomers were in peak 3 of Fig. 2a and that the other isomer was in peak 4. The isomers in peak 3 were completely desensitized to FBP but the isomer in peak 4 was sensitive to FBP. What was important was that only one of the three isomers had intact FBP-binding site. Therefore, it was reasonable to assign these hybrids to peaks 3 and 4 as in Fig. 2c.

The kinetic properties of other hybrids, with their own unique structural arrangements, also can be interpreted solely by the assumption that the FBP-binding site has a “dominant negative” nature. That D3W1 was completely desensitized indicated that the odd FBP-binding site composed of W and D subunits did not work. On the other hand, the strong activation of D1W3 by FBP indicated that the enzyme can be activated if there is one intact FBP-binding site. The side chains of Arg-173 and His-188 surround FBP from both subunits and interact with the phosphate moieties in the crystal structures (5, 6). Hence, the replacement of R173Q/H188Y on one side must destroy the integrity of the binding site.

The difference in the affinity between D2W2-QR and D2W2-P for the column may be accounted for the different location of charged tags on the enzyme, since no difference was observed on native PAGE or in the CD spectra. The distance between tags for D2W2-P, D2W2-Q, and D2W2-R hybrids were 45, 70, and 80 Å, respectively (see Figs. 1 and 2c). Negative charges were closer together in the D2W2-P hybrid than in the other two hybrids. This difference could explain the stronger affinity of D2W2-P for the anion exchanger. On column chromatography, only a part of the enzyme surface could touch the matrix.

**Allosteric Activation Mechanism of LDH**—We earlier proposed a model (6) for the activation mechanism of BLDH based on the crystal structures of the T- and R-states coexisting.
in the same crystal lattice. In that model, (i) FBP neutralizes the positive charge repulsion at the P-axis interface. (ii) The conformational change at the interface triggers a quaternary structural change to the R-state. (iii) The active site at the Q-axis subunit interface is affected by this quaternary structural change and takes on an active conformation. The quaternary structural change was thought to fulfill the concerted model of Monod et al. (9) in which the tetramer can contain either four T-state subunits (no affinity to substrate) or four R-state subunits (high affinity).

Hybridization experiments support the validity of this model. D-W hybrids could be classified into three types on the basis of their homotropic and heterotropic activation patterns. Each type corresponded to the number of intact FBP-binding sites. The second type had a sigmoidal saturation curve for pyruvate even in the presence of FBP. This property can be accounted for by the presence of both functional and nonfunctional FBP-binding sites in these two hybrids. The sigmoidal saturation curve indicated that the T-state was still dominant to the R-state in the presence of FBP. Charge repulsion at the P-axis interface seems to be partially neutralized by one FBP and the shift of equilibrium probably remains in an intermediate state.

The D-I hybrid experiments about enzyme activation by FBP showed inter-subunit allosteric communication via the Q-axis interface at a distance of about 40 Å. This result indicates that allosteric activation is mediated by a quaternary structural change. The quaternary structural change at the Q-axis interface has actually been observed by crystal structure analysis of BLDH (6). This structural change is accompanied by helix sliding at the Q-axis interface and subsequent positional replacement of His-68 of the next subunit along the Q-axis by Arg-171, one of the active-site residues, giving R-state. In the case of LDH from Bacillus steatorrhophilus, an allosteric mechanism caused by intra-subunit structural change is proposed (17). In that model, the binding of FBP causes Arg-173 to move and helix α2F to shift so that positioning of two adjacent residues, Arg-171 and Asp-168, that form the active site is improved. However, BLDH clearly does not act in this way because such an intra-subunit tertiary structural change could not explain the allosteric communication between the Q-axis-related subunits.

Enzyme activation with FBP caused S0.5 of both D-W and D-I hybrids to decrease, indicating that the hybrids retain the Km type allosteric properties (9) of the wild-type enzyme. The concerted allosteric model is interpreted on the basis of the Km type system (9), but not on that of the Vmax type system. The increase in Vmax accompanying activation of D-I hybrids with FBP, however, were about 2-fold (Fig. 5, b and c). There seems to be some additional mechanism by which the Vmax of the enzyme is increased in the presence of FBP. In this process, the inter-subunit transmission of activation is probably also involved, since Vmax of the D subunits changed when FBP bound to the I subunits.

In the D-I hybrid experiments, as the number of D subunits decreased, the Hill coefficient decreased, approaching 1 (Fig. 6) and S0.5 also decreased (Fig. 4). The decrease in the Hill coefficient agrees well with the allosteric model, but the behavior of S0.5 is contradictory. If the D-I hybrids keep the strict concerted model and have the same allosteric equilibrium constant, L (L = (enzyme in T-state)/enzyme in R-state) in the absence of substrate), as the wild type, S0.5 should increase as the number of active D subunits decrease (18). We suppose that the discrepancy arises because of the incorporation of I subunits into the hybrids, shifting the equilibrium toward the R-state. Replacement of His-195, which is near the Q-axis subunit interface, with asparagine may affect the equilibrium.