Immunological Study of Lactate Dehydrogenase from *Streptococcus mutans* and Evidence of Common Antigenic Domains with Lactate Dehydrogenases from Lactic Bacteria

PASCAL SOMMER,* JEAN-PAUL KLEIN, JOELLE A. OGIER, AND ROBERT M. FRANK

Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Faculté de Chirurgie Dentaire, 67000 Strasbourg, France

Received 6 June 1985/Accepted 17 September 1985

Rabbit polyclonal antibodies directed against purified *Streptococcus mutans* L-(+)-lactate dehydrogenase reacted with the purified enzyme, giving a marked deviation of its kinetic parameters. The enzyme affinity for pyruvate or NADH decreased in the presence of antibody, the affinity for fructose 1,6-diphosphate (FDP) appeared to be slightly affected, and the cooperativity of the ligand binding was lowered. A partial protective effect was observed when the enzyme was preincubated with FDP prior to the antibody adjunction. An enzyme-linked immunosorbent assay allowed demonstration of a 30% decrease in enzyme-antibody fixation when FDP was added. The protective effect observed with FDP could be correlated with a conformational change induced by the activator. A decrease of antibody binding in the presence of FDP was also obtained with *S. sanguis*, *Actinomyces viscosus*, and *Lactobacillus casei* lactate dehydrogenases, which reflects a similar mechanism of activation among lactic bacteria. NADH did not offer any protection against antibody inhibition or fixation, and the coenzyme affinity decrease could be attributed to an indirect mechanism. On the contrary, pyruvate and the immunoglobulins apparently could compete for specific binding sites. A decrease of antibody binding was also obtained with three heterologous lactic bacterial lactate dehydrogenases, indicating a conservation of antigenic determinants implicated in the substrate binding.

Lactic bacterial L-(+)-lactate dehydrogenase (LDH; EC 1.1.1.27), a key enzyme in fermentation metabolism, exhibits its structural and kinetic parameters which are closely related (10). Enzymes are generally activated by fructose 1,6-diphosphate (FDP). They consist of four identical subunits, with two or more binding sites for each ligand. A significant degree of similarity exists between their active centers, at least for the *Lactobacillus casei* (14) and the *Streptococcus cremoris* (7) enzymes, when respectively compared with vertebrate enzymes. Immunological cross-reactions between extragenera lactic bacterial LDHs revealed antigenic relationships (21), confirming the structural conservation of these molecules.

The LDH from *S. mutans* OMZ 175, a cariogenic strain (11), was recently purified to homogeneity (21). FDP exerts its activation effect by increasing the *S. mutans* OMZ 175 enzyme affinity for pyruvate and by lowering the cooperativity between each binding site as in the case of several bacterial LDHs (10). Reciprocally, pyruvate raises the affinity for FDP. The mechanism of this allosteric regulation has not yet been elucidated for the streptococcal enzymes. However, biophysical investigations of the *L. casei* FDP-dependent LDH (13) allowed detection of conformational changes associated with the effector binding. The aim of this work was to analyze the structural modifications observed after binding of FDP and substrates on *S. mutans* LDH. We used specific polyclonal antibodies directed against the purified enzyme to detect the consequence of antibody inhibition, with or without preliminary incubations with ligands, as previously performed for vertebrate LDHs (6). The effect of ligand binding on the antibody fixation upon the LDHs from *S. sanguis*, *L. casei*, and *Actinomyces viscosus* was also investigated.

MATERIALS AND METHODS

Organisms, culture conditions, and enzyme purification. *S. mutans* OMZ 175 (serotype f, 3, 19), *S. sanguis* OMZ 9, *A. viscosus* NY.1, and *L. casei* ATCC 4646 were previously described (20, 21). Bacteria were prepared from exponential-phase cultures grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.).

The *S. mutans* LDH cytoplasmic fractions were purified to homogeneity by DEAE-Sephacel and Blue-Sepharose CL-6B chromatographies and S200 Sephacryl gel permeation (21). *S. sanguis*, *A. viscosus*, and *L. casei* LDHs were only chromatographed on DEAE-Sephacel columns as previously reported (21). The enzymes were dialyzed against 20 mM Tris-maleate buffer, pH 6.2 (TM buffer), supplemented with 25% (vol/vol) glycerol and 0.01% (wt/vol) sodium azide (TMG buffer).

Antibody preparation. Antibodies to *S. mutans* OMZ 175 LDH were obtained as described elsewhere (21). Antibodies were precipitated with ammonium sulfate at 50% saturation and dialyzed against TM buffer. The immunoglobulin G (IgG) fractions were purified by chromatography on protein A-Sepharose CL-4B and dialyzed against TM buffer (21). Preimmune sera were obtained before rabbit immunization, and the IgG fractions were obtained as described above.

Antibody specificity was checked by crossed-immunoelectrophoresis (24) and Western blotting procedures (23) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Enzyme assay and inhibition experiments. LDH activity and the kinetic parameters were determined as previously described (21). Kinetic saturation curves for increasing concentrations of pyruvate, NADH, and FDP were plotted by the method of Hill as described previously (2, 22). Experimental data points from three experiments were adjusted by least-squares curve fitting.

The antibody-mediated inhibition level was determined...
FIG. 1. Crossed-immunoelectrophoresis of *S. mutans* crude extract against anti- *S. mutans* LDH antibodies. The well contained 3.7 µg of *S. mutans* cytoplasmic extract which was electrophoresed for 2.5 h at 2.5 V/cm. Antigens were then allowed to react with antibodies (0.15 mg/ml) raised against the purified LDH from *S. mutans* OMZ 175. Precipitates were detected by Coomassie blue R250 staining.

after preincubation of increasing amounts of antibody with *S. mutans* LDH. After 20 h at 4°C, each assay containing antibody and enzyme (2.6 µg) was tested for LDH activity.

The inhibition effect on the kinetic parameters was determined after preincubation of antibodies at different LDH concentrations. After 20 h at 4°C, aliquot fractions of the reaction mixtures containing 2.6 µg of LDH were tested for LDH activity by varying the concentration of one ligand. The concentrations of fixed ligands were 50 mM for pyruvate and 0.5 mM for NADH, and a concentration of 1.65 mM FDP was chosen to allow maximum activation (21).

Each inhibition assay included an analogous experiment using preimmune IgG. The percentage of residual activity in the presence of specific antibody was expressed by reference to the activity found in the presence of preimmune IgG.

Enzyme-linked immunosorbent assay. Purified *S. mutans* OMZ 175 LDH was dialyzed and diluted up to a protein concentration of 11.5 µg/ml in 0.1 M Tris hydrochloride buffer, pH 7.2. The enzyme (50 µl) was applied to each well of microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). After incubation for 17 h at 37°C, the plates were washed with 0.1 M Tris hydrochloride buffer, pH 7.2, containing 0.05% (vol/vol) Tween (THT buffer) and saturated with 3% (wt/vol) bovine serum albumin in THT buffer for 1 h at 37°C. The plates were then washed again, and 50 µl of anti-*S. mutans* LDH antibody (12.5 µg/ml) in THT buffer, corresponding to the minimum saturating amount of IgG previously predetermined (21), was added. Incubation of the plates for 2 h at 37°C was then performed, and the plates were then washed five times with THT buffer. The antibody binding was detected with goat alkaline phosphatase-labeled anti-rabbit antibody (Miles Laboratories, Inc., Elkhart, Ind.) diluted 1:500 in THT buffer. Finally, after 1 h at 30°C, the plates were washed again and a p-nitrophenyl-phosphate substrate was added at 1 mg/ml in 1 M diethanolamine buffer, pH 9.6. The increase in optical density at 405 nm was monitored after 2 h with a Multiskan Photometer (Flow Laboratories, Inc., McLean, Va.).

The effect of substrates on *S. mutans* antibody recognition was analyzed by adding increasing amounts of pyruvate, NADH, and FDP together with saturating amounts of anti-LDH antibody (12.5 µg/ml). The effect of ligands on heterologous antigen-antibody recognition was performed in a similar manner, but partially purified LDHs from *S. sanguis*, *A. viscosus*, or *L. casei* were substituted for the *S. mutans* LDH. The plates were coated with a saturating protein concentration of 300 µg/ml (with respect to anti-*S. mutans* LDH antibody) when the *S. sanguis* enzyme extract was used and with 500 µg/ml for the two others.

The results are expressed as the percentage of binding inhibition of anti-LDH antibody to homologous or heterologous antigens when ligands were added: \([1-\left(\frac{OD_i - OD_{0b}}{OD_d - OD_{0b}}\right)] \times 100\), where OD is the optical density at 405 nm obtained in the presence of ligand, OD is the optical density at 405 nm obtained without ligand, and OD and OD are the optical densities at 405 nm obtained by using preimmune IgG, without (OD) or with (OD) ligand.

Protein determination. Protein concentrations were determined by the method of Lowry et al. (17).

RESULTS

Specificity of antibodies and antibody-mediated inhibition of *S. mutans* LDH. The specificity of anti-*S. mutans* LDH IgG was tested by crossed-immunoelectrophoresis, which yielded a single precipitin line against a crude cytoplasmic extract from this organism (Fig. 1). Two antigens were dissociated in the first dimension at pH 8.3; however, the fused precipitin line obtained by immunoprecipitation indicates a reaction of identity between the two components (24). The two electrophoretic forms may be ascribed to monomeric or tetrameric states of the enzyme, as already described for *L. casei* LDH (12). On the other hand, the antibodies reacted only with a single protein in crude cytoplasmic extracts from the different bacteria after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to nitrocellulose paper (21).

Effect of antibody on kinetic parameters of *S. mutans* LDH. Modifications of the enzyme kinetic constants were determined after incubation of the purified LDH (2.6 µg) with anti-LDH antibodies. The amounts of IgG during the preincubation stage were 125 and 225 µg, which allowed inhibi-

<table>
<thead>
<tr>
<th>Variable ligand</th>
<th>Antibody</th>
<th>Amt of antibody (µg)</th>
<th>Apparent Hill coefficient (n_Hill)</th>
<th>Half-saturating concn (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDP None</td>
<td>Preimmune IgG</td>
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<td>1.2</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
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<td>0.9</td>
<td>0.012</td>
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<tr>
<td></td>
<td>Specific IgG</td>
<td>225</td>
<td>0.6</td>
<td>0.032</td>
</tr>
<tr>
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<td>225</td>
<td>1.1</td>
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<tr>
<td></td>
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<td>0.8</td>
<td>12.0</td>
</tr>
<tr>
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<td>1.8</td>
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<td></td>
<td>Specific IgG</td>
<td>225</td>
<td>1.1</td>
<td>0.30</td>
</tr>
</tbody>
</table>

* Purified *S. mutans* LDH was preincubated for 20 h at 4°C with anti-*S. mutans* LDH IgG fraction or preimmune IgG fraction. The kinetic parameters were then determined for aliquot fractions containing 2.6 µg of enzyme. The standard assay in 20 mM Tris-maleate buffer (pH 6.2) was used except that the concentration of one ligand was varied. The concentrations of fixed substrates were 50 mM for pyruvate, 0.5 mM for NADH, and 1.65 mM for FDP.

* Apparent Hill coefficients and half-saturating concentrations (S0.5v and M0.5v values) were determined graphically from Hill plots.
The initial increase in behavior. The mutans of the after V0L. 51, determination by anti-rabbit detected ligands. None ........................81, ug/ml; 0), reference Pyruvate. o.0.0.0.0,0.0.0.0.0.LDH Ligand added (mM) activity (%) ± SD

None ........................................ 81 ± 2.7

Pyruvate

1 ................................. 82 ± 1.4

5.8 ................................ 83 ± 2.8

30 .................................... 79 ± 0.7

NADH

0.08 ........................................ 89 ± 3.1

0.19 ..................................... 83 ± 4.2

0.30 .................................... 96 ± 9.2

FDP

0.09 ........................................ 67 ± 7.5

0.50 ..................................... 58 ± 9.4

1.65 .................................... 54 ± 3.0

* Purified S. mutans LDH was preincubated with the indicated concentrations of pyruvate, NADH, or FDP. After 1 h at 4°C, anti-S. mutans LDH antibodies (IgG fraction) were added (700 μg). The residual activity was tested after 20 h at 4°C on fractions containing 2.6 μg of enzyme and expressed by reference to assays performed with preimmune IgG.

Effect of ligands on antibody-mediated inhibition. The S. mutans LDH was preincubated with FDP, pyruvate, or NADH for 1 h at 4°C before adjunction of specific or preimmune antibodies, and the mixtures were then incubated for 20 h at 4°C. Table 2 shows the residual activity of assays containing 2.6 μg of LDH and various amounts of ligands. In controls without any ligand, 80% inhibition of the original enzymatic activity was observed when 700 μg of anti-S. mutans IgG was added. Addition of FDP allowed a 25% decrease in inhibition, resulting in 54% activity inhibition with 1.65 mM FDP. Pyruvate had no detectable effect on enzyme inactivation, while NADH slightly enhanced the inhibition.

Effect of ligands on antibody binding. An enzyme-linked immunosorbent assay procedure was used to assess the effect of ligands on the antigen-antibody recognition. The S. mutans LDH was coated in wells of a polystyrene microtiter plate, and the amount of fixed antibody was monitored as described in Materials and Methods. A possible decrease of the antigen-antibody recognition was analyzed by addition of increasing concentrations of each ligand to the antibody mixture. A maximal inhibition of 30% of antibody binding was observed with concentrations of FDP higher than 0.5 mM (Fig. 2A). An inhibition of 15% was also obtained with concentrations of pyruvate higher than 30 mM (Fig. 2B). The coenzyme exhibited no interfering effect (data not shown).

The effect of inclusion of ligands was also checked in

| FIG. 2. Influence of FDP and pyruvate on enzyme-antibody recognition. The detection of ligand influence on antibody binding was detected by enzyme-linked immunosorbent assay. Wells were coated with 50 μl of S. mutans LDH (11.5 μg/ml; ●), S. sanguis LDH (200 μg/ml; ●), A. viscosus LDH (500 μg/ml; ▲), and L. casei (500 μg/ml; ▼). Anti-S. mutans LDH IgG fractions (12.5 μg/ml) were incubated for 2 h at 37°C with the enzymes in the presence or absence of various concentrations of FDP (A) or pyruvate (B). The enzyme conjugate goat anti-rabbit antibody (diluted 1:500) was incubated for 1 h at 30°C. Hydrolysis time 2 h. The inhibition percentage of antibody binding was determined by reference to experiments carried out under the same conditions with preimmune IgG fractions. | FIG. 2. Influence of FDP and pyruvate on enzyme-antibody recognition. The detection of ligand influence on antibody binding was detected by enzyme-linked immunosorbent assay. Wells were coated with 50 μl of S. mutans LDH (11.5 μg/ml; ●), S. sanguis LDH (200 μg/ml; ●), A. viscosus LDH (500 μg/ml; ▲), and L. casei (500 μg/ml; ▼). Anti-S. mutans LDH IgG fractions (12.5 μg/ml) were incubated for 2 h at 37°C with the enzymes in the presence or absence of various concentrations of FDP (A) or pyruvate (B). The enzyme conjugate goat anti-rabbit antibody (diluted 1:500) was incubated for 1 h at 30°C. Hydrolysis time 2 h. The inhibition percentage of antibody binding was determined by reference to experiments carried out under the same conditions with preimmune IgG fractions. |
systems allowing the antibody recognition of heterologous enzymes. Instead of pure *S. mutans* LDH, active preparations from *S. sanguis*, *A. viscosus*, and *L. casei* were coated on the microtitration plates. FDP decreased the antibody binding by about 10% for the *S. sanguis* and *A. viscosus* extracts and 20% for the *L. casei* extract (Fig. 2A). On the other hand, an 8% reduction in antibody binding appeared when pyruvate was added during the recognition of the *S. sanguis* LDH by antibody. An 18% decrease in antibody binding was observed with the *A. viscosus* extract and one of 25% was seen with the *L. casei* enzyme (Fig. 2B). NADH did not offer any hindrance to antibody accessibility, as for the *S. mutans* LDH.

**DISCUSSION**

In a previous work, we showed that antibodies obtained against *S. mutans* OMZ 175 LDH recognized the native enzyme and led to almost complete inhibition of enzymatic activity (21). The binding of FDP partially reduces this inhibition by lowering the antibody accessibility (Table 2, Fig. 2A). However, antibodies do not inhibit the effector binding since no increase in the M_{0.5}V value for FDP is observed in the presence of antibody (Table 1). Therefore, the partial protection against the immunological neutralization of the enzyme must be caused by an indirect mechanism. It seems reasonable to assume that FDP induces conformational changes in the quaternary structure of the enzyme, as often described for regulatory molecules (6). This conformational change could be correlated with the enzyme activation process induced by FDP, since the concentrations of activator that allow the maximal activation (21) and the maximal protection of the enzymatic activity against antibody are in the same order. The possibility of a conformational change is confirmed by the drastic decrease of the effector binding cooperativity when antibodies are fixed. Thus, the FDP-induced conformational changes render some determinants unavailable for combination with antibodies, while the fixation of these latter molecules hinders the flexibility of the enzyme. The demonstration of binding of antibodies implicating the effector binding among heterologous LDHs allows a generalization of the concept of an activation process by conformational changes for the FDP-dependent LDHs tested. These observations broaden the previous findings on the *L. casei*, *L. curvatus* (18), and *S. faecalis* (25) LDHs in which the activation (18) or the thermal protection (25) induced by FDP is correlated with conformational changes of the three enzymes.

On the other hand, NADH does not offer any protection against antibody inhibition or fixation, as was the case for vertebrate LDHs which are not protected by their substrates (6). A direct competition between antibody and coenzyme binding does not occur, although competitions between the binding of antibody and NADH (or NADH) in favor of the coenzyme are often described for dehydrogenases (16). However, a decrease in the enzyme’s affinity for its coenzyme has been observed. This cannot be attributed to an indirect effect, resulting from inhibition of either FDP or pyruvate binding, since NADH binds independently (21), probably according to a compulsory binding order in which NADH is bound first, as for other bacterial (7, 12) or vertebrate (15) LDHs. Thus, it might be expected that the binding hindrance of NADH results from a "blockade" by steric hindrance or conformational change (6). As antibodies seem to block both the structural modification induced by FDP and the accessibility of the coenzyme by steric hindrance, it could be interesting to correlate the two mechanisms, as for the *L. casei* LDH, where spectroscopic measurements showed that the bound coenzyme was affected by the effector binding (18).

In spite of the lack of protection against immunological neutralization, pyruvate inhibits antibody fixation. The decrease of affinity for pyruvate observed in the presence of antibody does not result from an alteration of the FDP binding, since antibodies (i) do not reduce the enzyme affinity for the activator and (ii) lower the substrate binding cooperativity, while a decrease in the optimal effector concentration leads to a drastic lowering of the affinity of the enzyme for pyruvate and to an increase in the cooperativity of substrate binding (4, 8, 12, 21). This decrease results probably from a direct competition between the binding of pyruvate and antibody. The absence of any protective effect of pyruvate against the antibody inhibition might be attributed to the existence of two binding sites for the substrate, a noncatalytic binding site and a catalytic binding site, as described for *S. mutans* NCTC 10449 (4) and *L. casei* (12) LDHs. The antibody binding inhibition in the presence of pyruvate was also reported for the FDP-dependent *S. sanguis*, *L. casei*, and *A. viscosus* LDHs. The inhibition level of antibody fixation appeared higher when the number of common epitopes decreased: a 25% antibody binding inhibition was observed in the presence of pyruvate with the *L. casei* enzyme, which is the less immunologically related enzyme (21). This inhibition decreases with the *A. viscosus* LDH and appears very weak with the closely related *S. sanguis* LDH, as for the *S. mutans* enzyme. This appears strongly suggestive of a direct competition of antibodies for specific pyruvate binding sites which seem to be preserved by evolution. In this regard, it could be interesting to correlate this common antigenic determinant with the noncatalytic pyruvate binding site detected on the *L. casei* LDH (5). This latter region presented a sequential homology with the anion binding site of vertebrate LDHs (9, 14) onto which pyruvate binds (1), indicating a high degree of conservation. However, the use of polyclonal antibodies did not allow a direct demonstration of identity between the pyruvate binding site of bacterial LDHs and the vertebrate anion binding site, since no immunological cross-reaction could be observed between bacterial and vertebrate LDHs (21). The use of monoclonal antibody directed against the pyruvate binding domain might provide additional assertions on the regulation and evolution of bacterial LDHs.

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**LITERATURE CITED**