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# Immunochemical Evidence for Extensive Ligand-induced Conformational Changes in *Lactobacillus casei* Dihydrofolate Reductase*

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**The effects of ligand binding on antibody complex formation of *Lactobacillus casei* dihydrofolate reductase have been investigated. Binary complexes containing either substrate and inhibitors or $NADP^+$ and NADPH together with ternary complexes containing inhibitors and coenzyme were examined. Whereas substrate and inhibitor binding alone show no effect, the binding of coenzyme reduces antibody complex formation. The most striking effect is observed with ternary complexes containing methotrexate or aminopterin and NADPH: maximal retention of the labeled protein in the immunoprecipitation assay is reduced to ~30% of its original value with dihydrofolate reductase alone due to a decrease in both the affinity and lifetime of the antibody-protein complex at one or more antigenic sites. This result is discussed in terms of different conformational changes brought about by NADP and NADPH.**

The solution structure of globular proteins cannot generally be considered as rigid. Evidence for conformational flexibility has been obtained by a variety of methods such as hydrogen-exchange experiments (1), nuclear magnetic resonance spectroscopy (2), and fluorescence spectroscopy (3). This conformational mobility is in most cases essential for the functioning of proteins; for example, the binding of substrates according to the general "induced-fit" model as proposed by Koshland (4). Flexibility does not need to be limited to residues directly in contact with ligands, and for regulatory processes involving allosteric interactions or cooperative effects, long range conformational changes may be required. In the two classical models by Monod *et al.* (5) and Koshland *et al.* (6), describing cooperativity in multimeric proteins, the protein is assumed to exist in at least two conformational states, one of which becomes highly populated upon ligand binding. Examples for cooperativity between coenzymes and substrates or substrate analogues are found in dehydrogenases (7), and detailed measurements of cooperativity factors for the monomeric enzyme dihydrofolate reductase from *Lactobacillus casei* have been carried out (8, 9), indicating the existence of multiple conformations (10, 11).

We have used antibodies raised against native dihydrofolate reductase to probe the structure of binary and ternary complexes of *L. casei* dihydrofolate reductase via alterations in their antigenic reactivity. Assuming that the antibodies used bind exclusively to the native protein, any variation of this native conformation should affect its antigenic reactivity (12, 13).

## EXPERIMENTAL PROCEDURES

Dihydrofolate reductase was purified as in Ref. 14 and its concentration determined by assaying its catalytic activity. *L. casei* dihydrofolate reductase was kindly provided by G. Ostler (National Institute for Medical Research) and *Escherichia coli* dihydrofolate reductase by P. J. Cayley and R. W. King (National Institute for Medical Research, London). *N*-Bromosuccinimide modification of Trp 21 of *L. casei* dihydrofolate reductase was carried out as described in Refs. 15 and 16. Dansylation of *L. casei* dihydrofolate reductase was carried out according to Vehar *et al.* (17) and the modified protein was used immediately for experiments. $NADP^+$, NADPH, methotrexate, aminopterin, trimethoprim, and dihydrofolate were obtained from Sigma. All other chemicals used were of the highest purity commercially available.

Anti-*L. casei* dihydrofolate reductase antibodies were raised in adult female rabbits by standard immunization procedures: 500 μg of purified protein in complete Freund's adjuvant were injected intramuscularly at 10-day intervals into the legs and back of the animals and antiserum was subsequently prepared from weekly bleeds.

Immunoprecipitation was carried out using purified dihydrofolate reductase labeled with $^{125}I$ (18). Antibody binding was carried out by mixing aliquots of stock solutions of antibody and $^{125}I$-labeled dihydrofolate reductase for 30 min at room temperature in a reaction volume of 200 μl containing 150 mM NaCl, 50 mM Tris, pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (RIPA buffer). The protein-antibody complex was precipitated with 50 μl of a 10% solution of washed formalin-fixed protein A bearing *Staphylococcus aureus* (Cowan 1 strain), washed twice with 500 μl of 150 mM NaCl, 50 mM Tris-hydrochloride, pH 7.5, 5 mM sodium ethylenediaminetetraacetate, and 0.05% Nonidet P-40 (British Drug Houses, Ltd.), and the radioactivity in the pellet counted in a γ counter. $^{125}I$-labeled dihydrofolate reductase was used immediately following its preparation in order to ensure as little radiation damage as possible. Iodination does not affect the enzymatic properties of the protein over the period it was used (24 h) since the modified protein showed no significant alteration in its catalytic activity measured spectrophotometrically as described in Ref. 14. Spectrophotometric assays of catalytic activity were also carried out with dihydrofolate reductase stored in RIPA buffer and over a period of 1 h no significant change in activity could be detected.

The competitive radioimmunoassays were carried out at limiting antibody concentration, determined from a standard binding curve.

## RESULTS AND DISCUSSION

Fig. 1 shows standard antibody curves for serum from two animals, indicating the close similarity between both antibody populations. After initial testing for differences between these, with negative results, only one serum was used for the subsequent experiments. Table I summarizes the effects of ligand

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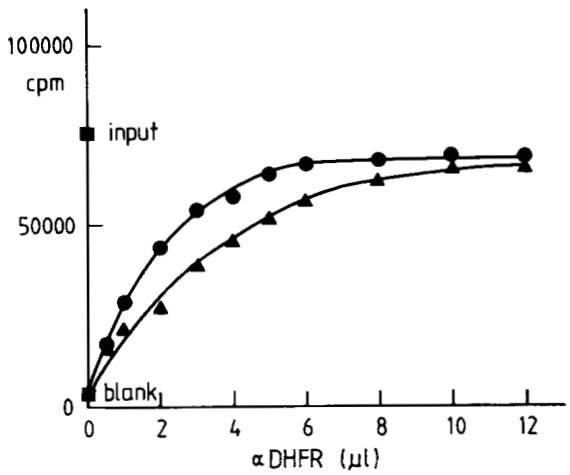


FIG. 1. **Binding curves obtained with serum containing anti-dihydrofolate reductase antibodies (*αDHFR*) from two different animals.** Anti-dihydrofolate reductase 1, ●; anti-dihydrofolate reductase 2, ▲. All subsequent experiments were carried out with anti-dihydrofolate reductase 1 serum. The experimental conditions are the same as those given in Table I except that the amount of anti-dihydrofolate reductase serum was varied.

TABLE I

*Effects of ligand binding on antibody complex formation of L. casei dihydrofolate reductase*

The experimental conditions were: 2 μl of $^{125}$I-labeled *L. casei* dihydrofolate reductase (~50 μM), alone or with ligand, were incubated with 4 μl of anti-dihydrofolate reductase serum in 200 μl of RIPA buffer; the concentrations of ligands employed were 50 μM methotrexate, 52 μM trimethoprim, 43 μM aminopterin, 45 μM dihydrofolate, 42 μM NADPH, and 94 μM NADP$^+$. All experiments were carried out in quintuplicate. Further details are given under "Experimental Procedures."

| Complex | *cpm*[a] |
|---|---|
| Dihydrofolate reductase alone | 41,300 (± 1,100) |
| + Methotrexate | 38,400 (± 2,000) |
| + NADP | 34,800 (± 2,500) |
| + NADPH | 22,800 (± 1,300) |
| Dihydrofolate reductase | |
| + Trimethoprim | 42,100 (± 2,300) |
| + NADP | 35,000 (± 2,300) |
| + NADPH | 35,600 (± 3,000) |
| Dihydrofolate reductase | |
| + Dihydrofolate | 42,000 (± 3,000) |
| + NADP | 34,600 (± 3,400) |
| + NADPH | 35,700 (± 1,900) |
| Dihydrofolate reductase alone[b] | 36,000 (± 2,000) |
| + Aminopterin | 31,200 (± 4,000) |
| + Aminopterin + NADPH | 12,700 (± 2,500) |

[a] Mean ± S.D.
[b] Separate experiment.

binding to *L. casei* dihydrofolate reductase on antibody complex formation. All experiments were carried out with antiserum amounts of 4 μl (~70% saturation with dihydrofolate reductase alone), and ligand concentrations were chosen to ensure >90% saturation of the enzyme with the ligand. Three features emerge from the data.

i. Inhibitor or substrate binding alone has no effect on antibody complex formation by dihydrofolate reductase. This demonstrates that the substrate binding site contains no antigenic site. Furthermore, substrate binding does not result in conformational changes involving other antigenic sites further removed from its own site.

ii. Coenzyme binding (both NADP$^+$ and NADPH) reduces the amount of dihydrofolate reductase in the immunoprecipitate to ~80% of its value without coenzyme, indicating either a loss of an antigenic site or a reduced affinity for a modified site. Loss of an antigenic site can occur either because the coenzyme occupies this site or by an induced conformational change in an antigenic site, brought about by coenzyme binding.

iii. Ternary complexes show effects that fall into two categories. Complexes with trimethoprim and either coenzyme and complexes with methotrexate or aminopterin and NADP$^+$ show the same reduction in the level of labeled protein immunoprecipitated observed with the coenzymes alone. However, complexes with methotrexate or aminopterin and NADPH show a large loss (~50%) in the level of labeled protein immunoprecipitated. This effect cannot be accounted for simply by blocking of the substrate and coenzyme binding site, since trimethoprim binds in the same binding site as methotrexate in dihydrofolate reductase (19). Furthermore both coenzymes show the same effect in binary complexes, but strikingly different effects in ternary complexes with methotrexate or aminopterin. Thus, the simplest explanation for this result is the occurrence of a substantial conformational change of the polypeptide chain of the native protein in a highly antigenic site in the case of the ternary complexes with methotrexate or aminopterin and NADPH. Support for this proposal comes from the experiment shown in Fig. 2 which compares the binding curve for native dihydrofolate reductase with the one for the ternary dihydrofolate reductase-methotrexate-NADPH complex. If the reduced immunoprecipitation of the ternary complex were simply due to a decrease in the affinity of the antibody for a modified antigenic site, one would expect a right shift in the binding curve with the maximal level of immunoprecipitated protein unaltered. In contrast, the experimental result shows a dramatic loss of ~70% in the maximal retention of labeled protein in the immunoprecipitate. This observation can only be accounted for by a large decrease in both the affinity and lifetime of antibody-protein complexes at one or more antigenic sites such that approximately 70% of the antibody-protein complexes dissociates during the precipitation of free antibody and antibody-protein complexes with protein A bearing *S. aureus*. Thus, the binding curve observed with the ternary

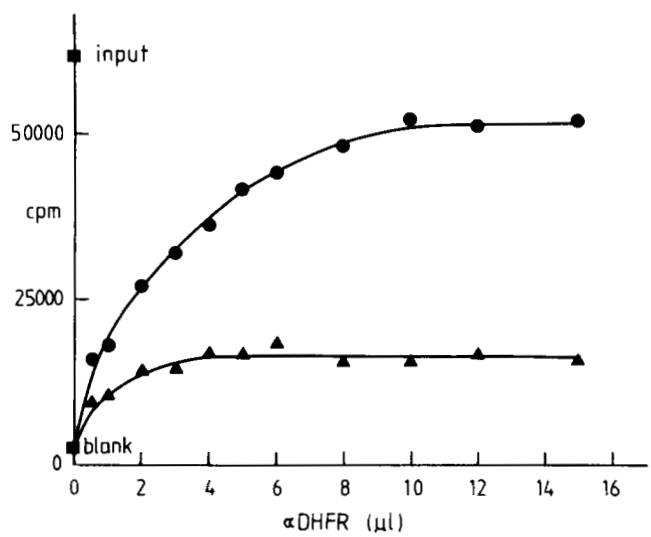


FIG. 2. **Binding curves for antibody binding of dihydrofolate reductase alone (●) and the dihydrofolate reductase-methotrexate-NADPH (▲) complex.** The experimental conditions are the same as those given in Table I except that the amount of anti-dihydrofolate reductase (*αDHFR*) serum was varied.

dihydrofolate reductase-methotrexate-NADPH complex is solely a manifestation of the remaining high affinity antigenic sites with long lifetimes unaffected by the presence of bound methotrexate and NADPH.

Ligand-induced conformational changes have been inferred from NMR studies on *L. casei* dihydrofolate reductase (20, 21) and it has also been suggested from fluorescence binding experiments that reduced coenzymes produce a change in part of the substrate binding site (9). Such a conformational change might contribute to the large cooperativity factor of 670 between methotrexate and NADPH (9). We therefore conclude that the loss in antibody binding in the presence of NADPH and methotrexate (or aminopterin) is due to a conformational change in the ternary complex which substantially alters the native protein structure and is responsible for the cooperative binding of NADPH and methotrexate to dihydrofolate reductase.

From a comparison of the crystal structures of the binary *E. coli* dihydrofolate reductase-methotrexate complex and the ternary *L. casei* dihydrofolate reductase-methotrexate-NADPH complex (22) it was suggested that the conformation of the polypeptide chain comprising residues 12-21 is altered by NADPH binding. However examination of the refined structures has now revealed that the changes are smaller than originally reported and could possibly be due to crystal packing (23). We cannot therefore propose with any certainty an involvement of this loop in the conformational change that destroys an antigenic site. One should, however, note that the comparison of the crystal structures involves dihydrofolate reductase from two different bacteria, and it might not be legitimate to undertake such a comparison since the influences of the different amino acid sequences on the precise folding of the polypeptide chain are not taken into account.

In order to establish if alterations of the active site have any effects on antibody binding of *L. casei* dihydrofolate reductase, the enzyme was modified selectively at Trp 21 using *N*-bromosuccinimide at a molar ratio of 4:1 as described previously (16). Using this modified enzyme in a competitive radioimmunoassay results in virtually the same competition as observed with unlabeled native enzyme (Fig. 3), thus indicating that Trp 21 and immediately adjacent amino acids in the active site are not part of an antigenic region.

Modification of a single lysine residue of *L. casei* dihydrofolate reductase with dansyl chloride (17), however, leads to a marked change in the competition experiment (Fig. 3). Up to concentrations of $\sim 2 \times 10^{-7}$ M unlabeled dansylated dihydrofolate reductase, no competition could be detected in contrast to the results with native dihydrofolate reductase and *N*-bromosuccinimide-modified dihydrofolate reductase where the same concentration of protein reduces the amount of $^{125}$I-labeled dihydrofolate reductase bound to the antibody to ~60–70% of its starting value. Moreover, at higher concentrations of protein the competition curve with dansylated dihydrofolate reductase is shifted to the right compared to the competition curves with native dihydrofolate reductase and *N*-bromosuccinimide-modified dihydrofolate reductase. This result indicates that modification of a single lysine residue probably abolishes a reactive antigenic site.

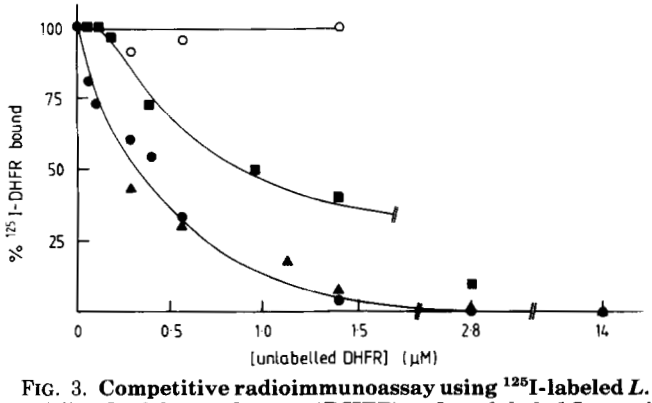


FIG. 3. **Competitive radioimmunoassay using $^{125}$I-labeled *L. casei* dihydrofolate reductase (*DHFR*) and nonlabeled *L. casei* dihydrofolate reductase (●), *N*-bromosuccinimide-modified *L. casei* dihydrofolate reductase (▲), dansylated *L. casei* dihydrofolate reductase (■), and *E. coli* dihydrofolate reductase form II (○).** Experimental procedure: aliquots of cold protein were added to a reaction mixture containing 2 μl of $^{125}$I-labeled *L. casei* dihydrofolate reductase (~50 μM) and 4 μl of anti-dihydrofolate reductase serum in 200 μl of RIPA buffer. Further details are given under "Experimental Procedures."

It has been argued that the lysine residue modified by dansyl chloride is either positioned in the active site or at a site which is changed by substrate and coenzyme binding in such a way as to bury the former reactive lysine (17). Given that dansylation of this lysine residue abolishes enzyme activity, that substrate and coenzyme binding leads to protection with respect to modification by dansyl chloride, and that the dansyl moiety is in close proximity to a tryptophan residue resulting in the quenching of enzyme fluorescence through energy transfer (17), it seemed likely that the specifically modified lysine residue was Lys 15. This was confirmed using [$^3$H]-dansyl chloride (24).

Intrigued by the close similarity of the crystal structures of *L. casei* and *E. coli* dihydrofolate reductase (23, 25) and aware of the fact that closely related proteins have been widely employed to investigate similarities in antigenic specificity and to determine the evolutionary trend of primary structure (13), we searched for immunochemical cross-reactivity between *L. casei* and *E. coli* dihydrofolate reductase. Previous studies with a different antiserum using Ouchterlony diffusion tests did not show any cross-reactivity between extracts of *E. coli* cells with and without a plasmid containing the *L. casei* dihydrofolate reductase structural gene (26). Since radioimmunoassays, however, are more sensitive than diffusion techniques it might have been possible to miss a small amount of immuno cross-reactivity. We therefore labeled *E. coli* dihydrofolate reductase (mixture of forms I and II) with $^{125}$I and used it with the anti-*L. casei* dihydrofolate reductase antibodies in the immunoprecipitation assay. In agreement with our previous findings (26), no binding above background was detected. The same result was obtained from a competition experiment as shown in Fig. 3. No competition between radiolabeled *L. casei* dihydrofolate reductase and unlabeled *E. coli* dihydrofolate reductase (form II) was found. This result might be somewhat surprising, considering that a primary antibody response to a globular protein is directed against its native three-dimensional structure (13). However, it has been pointed out that in globular proteins similarity in antigenic structure is not necessarily related to similarity in sequence (13) and our result leads to the related statement that similarity in the three-dimensional structure is also not necessarily related to similarity in antigenic structure. Clearly, both close similarity in sequence and three-dimensional structure are necessary requirements for immuno cross-reactivity.

Taking the known findings concerning antigenic regions in native globular proteins into consideration (13), namely that they are small (usually 6 or 7 residues), occupy surface locations, and interact primarily with the antibody through polar interactions (*e.g.* in the case of myoglobin, lysine is found in four of the five antigenic regions and the fifth contains arginine (13)) together with the results of the immunoprecip-

itation experiments, we propose that the position of the antigenic site in *L. casei* dihydrofolate reductase which is substantially altered by the cooperative binding of methotrexate and NADPH, is located at the the hairpin-turn which connects $\beta$ strand A to $\alpha$ helix B and contains Lys 15. Thus, upon modification of Lys 15 by dansyl chloride this site can no longer be efficiently recognized by antibodies directed toward it.

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