Lipid–Protein Interactions: Detergent Binding to L-Glutamic Acid Dehydrogenase

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Abstract □ A nonionic detergent was found to bind to the enzyme L-glutamic acid dehydrogenase [L-glutamate:nicotinamide adenine dinucleotide phosphate oxidoreductase (deaminating) EC1.4.1.3]. The amount bound was 17 moles of detergent/mole of enzyme, which, however, was not sufficient for the enzyme to be included in a detergent micelle.

Keyphrases □ L-Glutamic acid dehydrogenase—binding of nonionic detergent □ Detergents, nonionic—binding to L-glutamic acid dehydrogenase □ Binding—L-glutamic acid dehydrogenase to nonionic detergent □ Enzymes—L-glutamic acid dehydrogenase, binding to nonionic detergent

It has been reported (1) that the enzyme activity of L-glutamic acid dehydrogenase [L-glutamate:nicotinamide adenine dinucleotide phosphate oxidoreductase (deaminating) EC 1.4.1.3] changes in the presence of nonionic detergents. It was suggested that this change was due to some conformational event being produced in the enzyme by the detergent. Such a supposition implies that the detergent is bound to the enzyme. This paper reports an investigation of this hypothesis.

EXPERIMENTAL

The concentration of L-glutamic acid dehydrogenase solutions was determined using an $E^{1\%}_{280}$ of 10.0 (2). Quantitation of the enzyme in detergent solution and of ovalbumin was determined by fluorescamine (3).

Synthesis of ^{125}I-L-Glutamic Acid Dehydrogenase—The labeled compound was prepared by the method of Marchalonis (4) as modified by David and Reisfeld (5). To 600 μl of a 0.5% solution of enzyme in phosphate-buffered saline (1×10^{-4} *M* ethylenediaminetetraacetic acid, 0.1 *M* NaCl, and 0.04 *M* sodium phosphate, pH 7.4) were added 11 μg of lactoperoxidase coupled to 40 mg of wet Sepharose 4B[1] and 1 mCi of carrier-free ^{125}I-labeled sodium iodide. The reaction was initiated by addition of 20 μl of a 1×10^{-2} *M* solution of hydrogen peroxide in water.

The reaction mixture was shaken for a total of 10 min, but the shaking was stopped at 2-min intervals so that additional 20-μl aliquots of the hydrogen peroxide solution could be added. At the completion of the reaction, the radioiodinated enzyme was collected by centrifugation at 6000×*g* for 2 min. The supernate containing the enzyme was decanted off, and the beads were washed once with 0.5 ml of phosphate Buffer A (1×10^{-4} *M* ethylenediaminetetraacetic acid and 0.04 *M* sodium phosphate, pH 7.4) and centrifuged to extract any residual iodinated enzyme. The combined solutions were dialyzed overnight at 4° against 2 × 300-ml changes of phosphate Buffer A.

An equal volume of 2 *M* ammonium sulfate was added to the dialysate, and the solution was allowed to stand overnight at 4°. The precipitated protein was collected by centrifugation at 5000×*g* for 10 min, and the pellet was washed once with approximately 2 ml of chilled 2 *M* ammonium sulfate and recentrifuged. The pellet was dissolved in approximately 1 ml of phosphate Buffer A and dialyzed overnight at 4° against 2 × 300-ml changes of the same phosphate buffer.

The final traces of unbound iodine were removed by gel filtration on a column (2.3 × 9 cm) of Sephadex G-200. Fractions of 1 ml were collected and counted for radioactivity. Alternate fractions were assayed for protein by fluorescamine. Appropriate fractions, as determined by the ratio of radioactive counts to fluorescence, were pooled such that the specific activity of a preparation was typically 34,000 cpm/μg of enzyme.

Synthesis of ^{125}I-Nonionic Detergent—To 600 μl of a 10% solution of the detergent[2] in phosphate Buffer A were added 11 μg of lactoperoxidase coupled to 40 mg of wet Sepharose 4B and 1 mCi of carrier-free ^{125}I-labeled sodium iodide[3]. The reaction was initiated by addition of a 20-μl aliquot of a 1×10^{-2} *M* solution of hydrogen peroxide in deionized water. The reaction mixture was shaken for a total of 10 min, but shaking was interrupted at 2-min intervals so that additional 20-μl aliquots of the hydrogen peroxide solution could be added. A control was performed in an identical manner, except that 0.1 mCi of ^{125}I-labeled sodium iodide was used without lactoperoxidase or hydrogen peroxide.

After the iodination of the nonionic detergent, the entire reaction mixture was passed through a column (2.3 × 9 cm) of Sephadex G-15 to remove the unbound iodine. Fractions of 0.8 ml were collected and counted for radioactivity. The absorbance at 275 nm of alternate fractions was obtained; those fractions, as determined by the ratio of radioactive counts to absorption at 275 nm, were pooled such that a typical preparation with a specific activity of 8×10^9 cpm/mmole was obtained.

Micelle Formation and Binding of Nonionic Detergent to L-Glutamic Acid Dehydrogenase—*Sephadex G-75 Chromatography*—Samples (3.5–0.35%) of enzyme in phosphate Buffer A were mixed with equal volumes of radioiodinated nonionic detergent. The mixtures were incubated at 4° overnight and then passed through a column (0.9 × 14 cm) of Sephadex G-75 at 4°. The sample was eluted from the column using the phosphate buffer as eluant at a flow rate of 7 ml/hr. Each fraction of 0.3 ml was counted for radioactivity. A control consisting of only radioiodinated detergent was also run.

Bio-Gel A-15m Chromatography—A 50-μl aliquot of a 100-μg/ml solution of ^{125}I-labeled enzyme (specific activity of 7000 cpm/μg) was added to 100 μl of a 7% solution of enzyme in phosphate Buffer A. To this mixture was added 50 μl of detergent. A control was prepared in the same way, except that 50 μl of deionized water was substituted for the detergent.

The samples were then incubated at 37° for 15 min and then at 4° overnight. All samples were layered onto a column (0.9 × 14 cm) and eluted with phosphate Buffer B (0.2 *M* NaCl, 1×10^{-4} *M* ethylenediaminetetraacetic acid, and 0.04 *M* sodium phosphate, pH 7.4), which also contained 1% by volume of the nonionic detergent. Fractions of 0.2 ml were collected and counted for radioactivity.

Sephadex G-200 Chromatography—The molecular weight markers, L-glutamic acid dehydrogenase, and ovalbumin, each dissolved in Buffer B, were run through their respective columns (0.9 × 29 cm) to determine their elution volumes. The void volume of the columns also was determined with Blue Dextran 2000 with every column run. A 50-μl aliquot of a 60-μg/ml solution of ^{125}I-labeled enzyme (specific activity of 3.7×10^4 cpm/μg) in phosphate Buffer B was added to 100 μl of a 7% solution of enzyme in phosphate Buffer A. To this mixture was added 50 μl of the nonionic detergent. For the control, run under identical conditions, 50 μl of deionized water was added to the enzyme sample. For samples of radioiodinated enzyme containing the nonionic detergent, the eluting buffer also contained 1% by volume of the detergent. Fractions of 0.4 ml were collected and counted for radioactivity. Each peak was pooled and assayed for protein by the use of fluorescamine.

RESULTS AND DISCUSSION

The binding of a nonionic detergent to the enzyme L-glutamic acid dehydrogenase was investigated because such detergents previously enhanced the activity of the enzyme to an appreciable extent (1). This investigation necessitated the preparation of the radioiodinated detergent and the enzyme. Enzyme synthesis was achieved by the use of insolubilized lactoperoxidase (4, 5).

The interaction of the enzyme and detergent was studied using gel

[1] Worthington Biochemical Corp., Freehold, N.J.

[2] Triton X-102, Sigma Chemical Co., St. Louis, Mo. This detergent possessed a hydrophilic portion in the form of a polyoxyethylene moiety and a lipophilic portion consisting of octylphenyl. These data were obtained from Rohm and Haas Surfactants Technical Bulletin, Rohm and Haas Co., Philadelphia, Pa.

[3] New England Nuclear Corp., Boston, Mass.

filtration. The results of this procedure have been shown to be in good agreement with those obtained by equilibrium dialysis (6). However, this technique will not determine accurately the binding parameters of any rapidly equilibrating system; under such circumstances, an overestimation of binding is observed.

In the present studies, it was possible to separate the detergent bound to the enzyme from that unbound and to estimate the amount of interaction between the two moieties. The enzyme, at a 4–5% concentration dissolved in a 0.9% solution of radioiodinated detergent, was chromatographed on Sephadex G-75. It was found that 1.4×10^{-5} mmole (mol. wt. 744) of the detergent was associated with 825 μg of the enzyme. This result indicates that 17 moles of detergent bound per mole of enzyme, calculated on the latter having a molecular weight of 1×10^6. This ratio of detergent to enzyme nearly corresponds to one binding site per 55,000 molecular weight subunit of the enzyme (7). This calculation presupposes detergent that is bound rather than in the micellar form. Such binding has occurred when ligand and protein were present in relatively low concentrations (8–10).

One interesting aspect of the interactions between proteins and detergents is the inclusion of several protein molecules in one detergent micelle (11). To test whether the enzyme could be included in detergent micelles, solutions of the radioiodinated enzyme in detergent were prepared and passed through a column of Bio-Gel A-15m. The results indicated that the enzyme could not be aggregated in detergent micelles, because no sample of L-glutamic acid dehydrogenase in detergent had an elution volume less than the native enzyme, even when the eluting buffer contained 1% by volume of the detergent.

While this experiment on agarose showed that the enzyme was not measurably aggregated in detergent micelles, it was not clear whether the enzyme was dissociating into its 55,000 subunits (7). Therefore, gel filtration on Sephadex G-200 was performed. The enzyme buffer only eluted as a single peak, whereas the enzyme in detergent gave a chromatograph of two peaks. In this latter case, the first peak eluted with the void volume whereas the position of the second peak indicated a molecular weight of 55,000. However, the amount of dissociation into these small subunits was never more than 5% of the total amount applied to the column.

The results indicate that the nonionic detergent can bind to the enzyme L-glutamic acid dehydrogenase. This binding is only in the order of approximately 17 moles of detergent/mole of enzyme, hardly sufficient to allow the enzyme to be included in a detergent micelle. However, it obviously is sufficient to cause enhancement of the activity of this enzyme (1).

These results suggest a possible avenue for enhancement of the biological activities of other proteins and drugs.

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Separation and Quantitative Determination of 2,4-Disulfamyl-5-trifluoromethylaniline in Hydroflumethiazide Using High-Pressure Liquid Chromatography

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Abstract □ A high-pressure liquid chromatographic method was developed to quantify 2,4-disulfamyl-5-trifluoromethylaniline in hydroflumethiazide using a bonded pellicular packing. An internal standard was added to the samples to ensure reproducibility. The results were compared with methods currently in use.

Keyphrases □ 2,4-Disulfamyl-5-trifluoromethylaniline—high-pressure liquid chromatographic analysis in hydroflumethiazide samples and tablets □ Hydroflumethiazide—prepared samples and tablets, high-pressure liquid chromatographic analysis of its precursor 2,4-disulfamyl-5-trifluoromethylaniline □ High-pressure liquid chromatography—analysis, 2,4-disulfamyl-5-trifluoromethylaniline in hydroflumethiazide prepared samples and tablets □ Antihypertensives—hydroflumethiazide prepared samples and tablets, high-pressure liquid chromatographic analysis of its precursor 2,4-disulfamyl-5-trifluoromethylaniline

The hydroflumethiazide monographs in the National Formulary (1) and the British Pharmacopoeia (2) include assays for free amine in the form of its precursor 2,4-disulfamyl-5-trifluoromethylaniline (I) (3–6). In the BP method, the free amine in the sample is compared visually with a standard of I using TLC for the separation. A diazotization is performed in the NF method, and the solution absorbance is read spectrophotometrically and compared with a standard preparation.

This paper describes a high-pressure liquid chromatographic (HPLC) method for the separation of I from hydroflumethiazide, and the results are compared with those from the compendial methods.

EXPERIMENTAL

Materials—Standard solutions containing 0.02, 0.04, 0.06, and 0.08 mg of I[1]/ml of reagent grade methanol were prepared. Hydroflumethia-

[1] E. R. Squibb standard substance.