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PURIFICATION OF CYTOPLASMIC ALDEHYDE DEHYDROGENASE BY COVALENT CHROMATOGRAPHY ON REDUCED THIOPROPYL-SEPHAROSE 6B

TREVOR M. KITSON*
Department of Biochemistry, University of Hull, Hull HU6 7RX (Great Britain)
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SUMMARY

Disulfiram-modified cytoplasmic aldehyde dehydrogenase binds covalently to the thiol groups of reduced thiopropyl-Sepharose 6B under conditions in which mitochondrial aldehyde dehydrogenase does not bind. After washing the resin, the uncontaminated form of the enzyme is eluted by dithiothreitol solution.

INTRODUCTION

Previous studies[1] have shown that the cytoplasmic aldehyde dehydrogenase of sheep liver is very much more sensitive to the inactivatory effect of disulfiram than is the mitochondrial form of the enzyme. (Disulfiram, or tetraethylthioperoxydicarbonic diamide, is a drug used in the treatment of chronic alcoholics[2].) However, detailed interpretation of the results of studies with the cytoplasmic enzyme has been complicated by the fact that published isolation procedures for this enzyme lead to material contaminated to a significant extent with mitochondrial aldehyde dehydrogenase[3]. The idea behind the present work was to utilise the pronounced specificity of disulfiram for cytoplasmic aldehyde dehydrogenase as a means of purifying this enzyme from contamination by the mitochondrial species. Thus it was proposed that the disulfiram-modified enzyme would bind covalently to an insoluble resin carrying thiol groups whereas other proteins (including the mitochondrial enzyme) would not bind. After washing the resin, pure cytoplasmic aldehyde dehydrogenase would be eluted by the reductive action of a low-molecular-weight thiol, such as dithiothreitol. As discussed below this proposal was shown to be correct.

As well as furnishing a method for removing mitochondrial contamination, the present study is of theoretical interest from the points of view of the versatility of covalent affinity chromatography and of the nature of the disulfiram-reactive groups in cytoplasmic aldehyde dehydrogenase. Moreover, the technique may lead to identification of the amino acid residues within the enzyme which carry the disulfiram-sensitive thiol groups.

* Present address: Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North, New Zealand.

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EXPERIMENTAL

Materials

NAD^+ was purchased from Boehringer (London, Great Britain). Disulfiram and dithiothreitol were obtained from Sigma (London, Great Britain). Thiopropyl-Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). All other chemicals were analytical-reagent grade whenever available, purchased from Fisons (Loughborough, Great Britain) or BDH (Poole, Great Britain). Solutions of acetaldehyde were made up daily from 1 *M* stock solutions (kept frozen), which were prepared from freshly redistilled acetaldehyde.

Methods

Protein concentrations. For purified cytoplasmic aldehyde dehydrogenase, protein concentration was determined spectrophotometrically using a specific extinction coefficient at 280 nm of $A^{1\%}_{1cm} = 11.3$.

Enzyme assay. This was performed fluorimetrically as described by Hart and Dickinson[4].

Preparation of sheep liver aldehyde dehydrogenase. The preparation of the cytoplasmic enzyme was carried out essentially by the procedure of Crow *et al.*[5]. Dickinson and Berrieman[3] have shown that this method results in cytoplasmic aldehyde dehydrogenase which usually contains some contaminating mitochondrial enzyme, but which is otherwise pure. A sample of the mitochondrial enzyme (prepared by the method of Hart and Dickinson[4]) was a generous gift from Dr. G. J. Hart.

Reduction of thiopropyl-Sepharose 6B. This was carried out using 2-mercaptoethanol according to the instructions in the booklet *Thiopropyl-Sepharose 6B — immobilised thiol reagent*, available from the manufacturers.

The binding of disulfiram-modified aldehyde dehydrogenase to reduced thiopropyl-Sepharose 6B. All operations involving the enzyme were carried out at 0–4°C. All buffers contained 0.3 m*M* EDTA. Thiopropyl-Sepharose 6B (1 g dry weight) was reduced to the free thiol form and washed well with 20 m*M* sodium phosphate buffer, pH 8.0. The resin was centrifuged gently and excess buffer poured off. Aldehyde dehydrogenase was dialysed against the same buffer; to 4 ml of the resulting enzyme solution (8–15 mg/ml) was added 20 μl of freshly made 20 m*M* disulfiram in ethanol. The enzyme activity was assayed before and after the addition of disulfiram. The disulfiram-treated enzyme solution ws added to the resin prepared as above, the resin was dispersed gently with a glass rod, and the mixture was allowed to stand for *ca.* 4 h with occasional gentle stirring. The resin was then lightly compacted by centrifugation, the supernatant was decanted and the resin was washed thoroughly with several changes of 20 m*M* phosphate buffer, pH 8.0, by dispersal with a glass rod, centrifugation and decantation. The resin was then dispersed in 3 ml of 20 m*M* dithiothreitol in 40 m*M* phosphate buffer, pH 8.0, and left overnight. After centrifugation, the eluted aldehyde dehydrogenase solution was collected by decantation. (In calculating the recovery of enzyme activity from the resin, the volume of the decanted solution was corrected for the volume of solution contained in the compacted wet resin; 1 g dry weight of resin occupies 3 ml when wet.)

Attempted binding of disulfiram-modified aldehyde dehydrogenase to reduced thiopropyl-Sepharose 6B on a large scale. A preparation of sheep liver cytoplasmic

aldehyde dehydrogenase was carried out to just after the first $(NH_4)_2SO_4$ fractionation stage, at which point the enzyme solution is grossly impure. The solution was dialysed against 40 m*M* sodium phosphate buffer, pH 8.0. To the enzyme solution (370 ml, 58 mg/ml) was added sufficient 4 m*M* disulfiram in ethanol (7.5 ml) to cause a substantial degree of inactivation (the remaining activity was 29% of the starting figure). Then 14 g (dry weight) of reduced thiol-resin (which had been washed with 40 m*M* phosphate buffer, pH 8.0) was added and the mixture was stirred gently for 4 h. (All buffers had been deaerated by standing under suction to minimise non-specific oxidative coupling of protein thiol groups to the resin.) The mixture was centrifuged and the supernatant decanted. The resin was thoroughly washed on a sintered-glass funnel with 40 m*M* phosphate buffer, pH 8.0, and then packed into a small glass column (15 × 2 cm I.D.). 15 m*M* Dithiothreitol in 40 m*M* phosphate buffer, pH 8.0, was then run through the column until it was just detectable in the eluate [from its effect on a sample of 5,5′-dithiobis-(2-nitrobenzoic acid)]. The column was left overnight, and the first 40 ml of eluate were then collected and assayed for enzymic activity.

RESULTS AND DISCUSSION

The concept underlying the experiments reported here is that there should be some way of using the highly specific reaction between disulfiram and cytoplasmic aldehyde dehydrogenase to furnish a purification of this enzyme from non-disulfiram-sensitive protein (such as the mitochondrial form of aldehyde dehydrogenase). With this view in mind, the feasibility of using the sequence of reactions shown in Fig. 1 was investigated. First is depicted the straightforward reduction of commercially available thiopropyl-Sepharose 6B to a resin with a high concentration of free thiol groups. Secondly, the reaction between enzyme thiol groups and disulfiram is shown. Previous work[6] has shown that this reaction is very rapid with the cytoplasmic enzyme and that no more than two molecules of disulfiram are required per tetrameric enzyme molecule for maximum inactivation; however, the mitochondrial enzyme only reacts significantly in the presence of high concentrations of disulfiram over a relatively long period of time[1,4]. Thus in a mixture of cytoplasmic aldehyde dehydrogenase with the mitochondrial enzyme (or other protein) only the former enzyme species should become modified when limiting amounts of disulfiram are added. Reaction 3 shows the reaction between reduced thiopropyl-Sepharose 6B and the disulfiram-modified enzyme, a reaction which theoretically should go to completion since the diethyldithiocarbamate ion is a good leaving group. At this stage non-covalently bound protein (including the mitochondrial enzyme) would easily be removed physically from the insoluble polymeric matrix. Elution of pure cytoplasmic aldehyde dehydrogenase would then be effected by reductive disulphide-interchange with an excess of a small molecular weight thiol such as dithiothreitol, as shown in reaction 4.

Of course, the disulfiram-modified cysteine residues in cytoplasmic aldehyde dehydrogenase might be sterically inaccessible to the resin's thiol groups, in which case reaction 3 would not proceed and the purification scheme would fail. Conversely, the reaction might go too far; reaction 5 shows one of the excess thiol groups on the resin displacing the bound enzyme. This would result in the reactivation of the di-

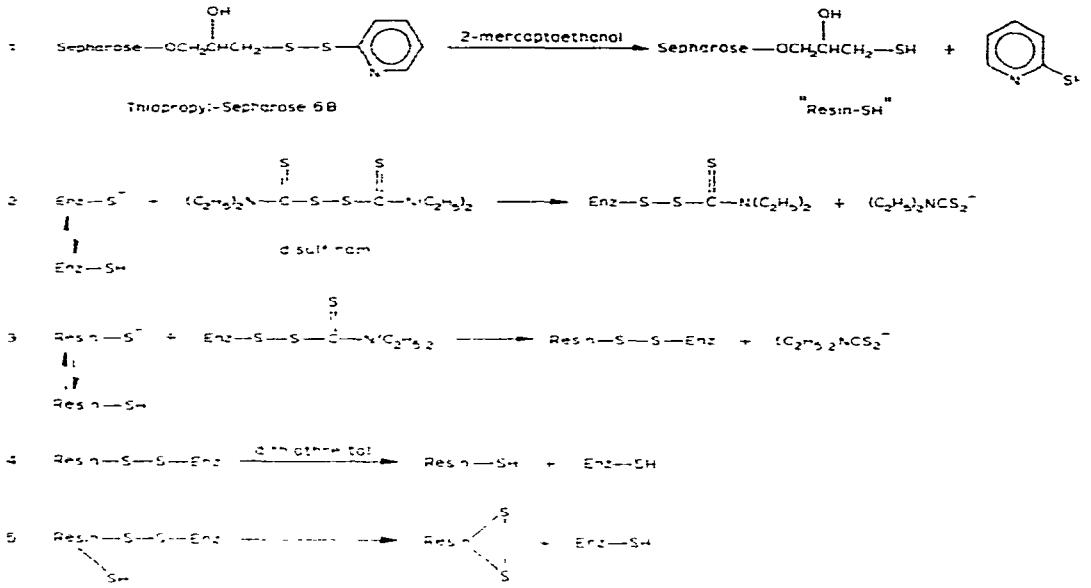

Fig. 1. The chemical reactions involved in the purification of cytoplasmic aldehyde dehydrogenase by covalent chromatography on reduced thiopropyl-Sepharose 6B.

sulfiram-modified enzyme, but this species would then find itself back in solution with the contaminating mitochondrial form or other impurities. (The sum of reactions 3 and 5 is analogous to the reactivation of disulfiram-modified cytoplasmic aldehyde dehydrogenase, which is brought about by high concentrations of a low molecular weight thiol, such as 2-mercaptoethanol[6].)

The results in Table I show to what extent the various possible reactions referred to above proceed in practice. Experiments A–C show that on a small scale there is moderate success in the amount of cytoplasmic aldehyde dehydrogenase which can be bound to and subsequently eluted from the thiol-resin (Between 35 and 44% of the units of activity which were abolished upon disulfiram-modification are recoverable from the resin.) A rather more variable amount of the disulfiram-modified enzyme is reactivated in solution, presumably according to reactions 3 and 5. The facts that under similar conditions virtually no mitochondrial enzyme is bound to the resin (Experiment E) and, in the absence of disulfiram-treatment, very little cytoplasmic enzyme is bound (Experiment D) confirm that the positive results in Experiments A–C must arise from operation of the reactions shown in Fig. 1 as predicted (and not through any non-specific binding by, for example, interaction of the resin's thiol groups with protein disulphide bridges).

When the large-scale purification of very impure aldehyde dehydrogenase was attempted using this method, no activity was recovered from the resin (Experiment F; repetition of the experiment gave the same result). Presumably the presence of large amounts of other proteins interferes in some way with the reaction between the thiol-resin and the modified enzyme. Even on a small scale using much purer aldehyde dehydrogenase the recovery of enzyme did not exceed 44%. Nevertheless, since the mitochondrial enzyme does not bind under these conditions (Experiment E), the method is useful and important in providing pure samples of cytoplasmic aldehyde

TABLE I

PURIFICATION OF CYTOPLASMIC ALDEHYDE DEHYDROGENASE BY COVALENT CHROMATOGRAPHY ON REDUCED THIOPROPYL-SEPHAROSE 6B

Enzyme activity was assayed as described under *Methods*; 1 unit is defined as the amount of enzyme catalysing the formation of 1 nmol of NADH per minute in the standard assay. The first four columns represent, respectively, (i) the total number of units of activity at the start of the experiment, (ii) the remaining activity after disulfiram treatment, (iii) the activity found in the supernatant after standing the disulfiram-modified enzyme in the presence of reduced thiopropyl-Sepharose 6B for 4 h, (iv) the activity which was eluted from the resin by dithiothreitol after having been covalently attached. "Recovery" is the total of columns 3 and 4 expressed as a percentage of column 1. (A large difference between "recovery" and 100% signifies disulfiram-modified enzyme which was neither bound to the resin nor reactivated in solution.) The last two columns represent, respectively, the activity which was covalently bound to the resin and the activity which reappeared in solution, expressed as a percentage of the number of units of activity which had been abolished by disulfiram treatment. Experiments A–C are small-scale experiments with the cytoplasmic enzyme; D is an analogous experiment without disulfiram treatment; E is an experiment analogous to A–C, but using the mitochondrial enzyme; F is a large-scale attempt using impure cytoplasmic enzyme.

Expt.	*Units of enzyme activity*				*Recovery (%)*	*Fate of the disulfiram-modified enzyme*	
	Original activity	*After disulfiram treatment*	*Supernatant*	*Bound and eluted*		*Bound and eluted (%)*	*Reactivated in solution (%)*
A	2072	456	953	563	73	35	31
B	2488	624	1536	826	95	44	49
C	1640	62	271	693	59	44	13
D	2004	–	1816	50	93	–	–
E	916	916	859	7	95	–	–
F	111,000	31,860	49,059	0	44	0	15

dehydrogenase for experiments in which mitochondrial enzyme contamination is unacceptable.

The purification of an enzyme by affinity chromatography usually relies on the affinity which the native enzyme has for some grouping which is covalently attached to an inert support. The affinity is often expressed in a non-covalent binding such as that between many dehydrogenases and AMP-Sepharose, but it may result in a covalent attachment such as that which occurs between papain or urease and activated thiol-Sepharose. The sequence of reactions in Fig. 1 (which we have seen is experimentally supported by the results in Table I) constitutes an unusual and interesting variation of affinity chromatography, partly because two separate affinities are involved and partly because it entails the deliberate inactivation of the enzyme which it is desired subsequently to purify. Thus initially, the affinity of cytoplasmic aldehyde dehydrogenase for disulfiram ensures that only this species becomes modified (reaction 2) and secondly, the affinity of the resin's thiol groups for the resulting reactive diethylthiocarbamoyl disulphide linkage ensures that such modified protein becomes bound to the resin (reaction 3).

The thiol groups on reduced thiopropyl-Sepharose 6B are separated from the polymeric matrix by a relatively short spacer group (see Fig. 1). Thus the fact that reactions 3 and 5 occur at all (and the results in Table I show that they both do to a substantial extent) must mean that the disulfiram-modified groups of cytoplasmic

aldehyde dehydrogenase are on or close to the enzyme's surface and not buried in some sterically inaccessible position within the enzyme molecule.

A further potential value of the work described here is that it allows the immobilisation of pure cytoplasmic aldehyde dehydrogenase specifically through the cysteine residues which react with disulfiram. This means that the sequencing of the peptide(s) containing the disulfiram-reactive cysteine residues may prove to be relatively simple. The isolation procedure would involve protease digestion of the immobilised enzyme, the washing away of free protease and interfering peptides, followed by the elution of the pure peptide(s) of interest by dithiothreitol solution. (The cysteine-containing peptides of human ceruloplasmin have been isolated from an activated thiol-Sepharose in this manner[7].) The importance of disulfiram to an understanding of the enzymology of aldehyde dehydrogenase would make this a desirable achievement.

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