Immobilized subunits can function as an affinity sorbent to purify oligomeric enzymes: the usefulness of hybridization on the solid support

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Summary. Immobilized dimers of yeast glyceraldehyde-3-phosphate dehydrogenase covalently bound to sepharose were shown to form hybrids with soluble dimers of the homologous enzymes present in crude tissue extracts (rat skeletal muscle, rat, rabbit and bovine hearts, rat liver, rat brain). Immobilized hybrid tetramers were then dissociated to form purified soluble enzymes.

The effectiveness of affinity chromatography as a method for protein purification[1] largely depends upon the specificity of the interaction between an immobilized ligand and a protein to be isolated. One of the most specific is the antigen-antibody interaction, but the use of matrix-bound antibodies as affinity sorbents has some disadvantages. Namely, a homogeneous preparation of the antigen must be available to obtain specific antibodies; purification of an enzyme is only possible after it has been isolated by some other procedure.

A similar situation apparently arises when isolated subunits of an oligomeric enzyme immobilized on a solid support are used to extract soluble subunits of the same protein from a multicomponent mixture[2]. Another approach to this problem is the use of a single sort of matrix-bound subunits for the isolation of several soluble enzymes differing in composition. This becomes possible with the technique of hybridization between homologous proteins. We demonstrated previously that matrix bound dimers of yeast glyceraldehyde-3-phosphate dehydrogenase (GAPD) obtained by dissociation of the immobilized tetramer readily formed hybrids with soluble dimers of the homologous rat muscle enzyme[3,4]. Here we report that this technique is applicable for isolation of GAPD from crude extracts of different mammalian tissues. The properties of the isolated enzymes can then be studied by investigating the characteristics of the dimeric species within the immobilized hybrid molecules; another approach is to solubilize the non-covalently bound dimers to obtain an individual enzyme preparation.

Methods. GAPD isolated from baker's yeast[5] was coupled with weakly CNBr-activated sepharose 4B as previously described[6]. Incubation of the immobilized tetrameric enzyme for 1 h at 4 °C in the presence of 0.15 M NaCl, 50 mM ATP, 5 mM EDTA, 2 mM dithiothreitol (pH 7.3) resulted in its complete dissociation into dimers. The matrix-bound dimeric species which remained active even in the presence of the dissociating agents were separated from the split inactive dimers by extensive washing with 0.1 M sodium phosphate, 5 mM EDTA, 2 mM dithiothreitol (pH 7.3).

To obtain crude tissue extracts, a portion of the corresponding tissue was homogenized in a 3-fold volume of 0.1 M sodium phosphate (pH 7.2) containing 5 mM EDTA, with subsequent centrifugation for 30 min at 40,000 × g. The suspension of sepharose that contained immobilized dimers of yeast GAPD, was then mixed with a given tissue extract. The amount of the latter was sufficient to provide a 3 to 30-fold excess of GAPD activity over that present in the matrix-bound form. The mixture was incubated for 60 min at 4 °C and then at 25 °C for 30 min. This was followed by extensive washing with 0.1 M sodium phosphate, 5 mM EDTA (pH 7.2) to remove soluble proteins.

Results and discussion. The table demonstrates that immobilized dimers of yeast GAPD are capable of forming hybrids with homologous enzymes present in crude extracts of various mammal tissues. The extent of reconstruction of the matrix-bound tetramers (77–89%) does not significantly differ from that observed in the case of reassociation of immobilized yeast dimers with the soluble enzyme of the same origin. The table also demonstrates that specific activity of the immobilized species is markedly higher that that of the enzyme in crude tissue extracts. These data are, however, insufficient to calculate the degree of enzyme purification, for the following reasons.

The yeast and muscle dimers comprising hybrid molecules exhibit different pH optima[3], and this results in their unequal contribution to the total hybrid activity assayed at pH 9.3. To evaluate only the muscle dimer activity, we prepared hybrid molecules containing the matrix-bound yeast dimers carboxymethylated at essential cysteine residues and therefore inactive[4]. Sp. act. of this preparation corresponded to 22 U/mg, which does not significantly differ from sp. act. of the matrix-bound tetrameric GAPD, obtained by the immobilization of the homogeneous crystalline enzyme[8] and assayed under comparable conditions.

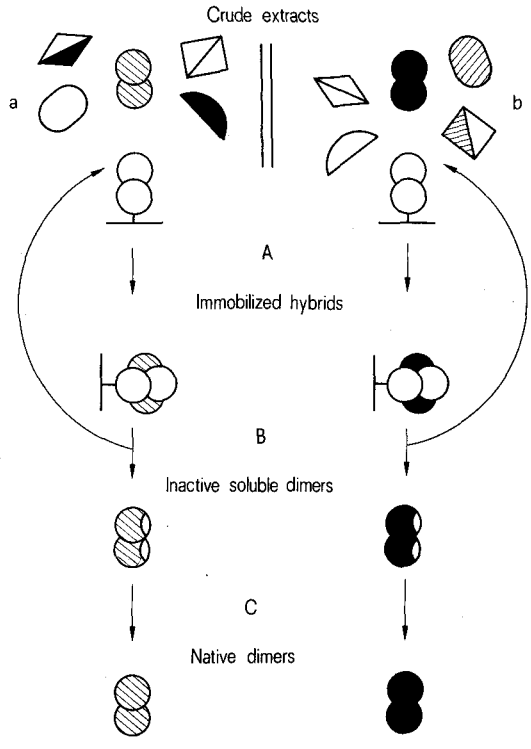
Hybridization between the immobilized dimers of yeast GAPD and soluble dimers of homologous enzymes present in crude tissue extracts

Preparation of matrix-bound GAPD	Immobilized protein content (mg/g dry sepharose)	%	Activity (U/g dry sepharose)	Sp. act. (U/mg immobilized protein)	Sp. act. in the tissue extract (U/mg protein)
Yeast tetramers	6.6	100	77.9	11.8	-
Yeast dimers	3.6	55	41.8	11.6	-
Hybrid tetramers					
Yeast-rat muscle	5.52	84	89.4	16.2	1.7
Yeast-rat brain	5.4	82	49.7	9.2	1.9
Yeast-rat liver	5.7	86	58.1	10.2	1.7
Yeast-rat heart	5.1	77	63.8	12.5	1.1
Yeast-bovine heart	5.88	89	79.4	13.5	2.0
Yeast-rabbit heart	5.7	86	74.1	13.0	1.9

The content of matrix-bound protein was determined spectrophotometrically in polyethylene glycol[7]. The enzymatic activity of the immobilized dehydrogenase was assayed at 25 °C using a SF-4 spectrophotometer. A 1 cm quartz cell contained 0.1 M glycine buffer (pH 9.3), 5 mM EDTA, 5 mM sodium arsenate, 1.5 mM glyceraldehyde-3-phosphate, 2 mM NAD and 0.1 ml of the immoblized enzyme suspension (2–5 μg protein) in the total volume of 3 ml. The reaction was started by the addition of substrate and followed for 15–20 sec.

This indicates that a sufficiently pure protein can be obtained by a one-step procedure outlined above (figure, A). It should be pointed out that the reassociation of the immobilized and soluble dimers proceeded under non-denaturating conditions, in the absence of any dissociating agent. This suggests the presence of dimeric forms of the enzyme in the crude tissue extracts. Tetrameric apo-GAPD was shown to dissociate reversibly into dimers on dilution[9]. The incomplete saturation with NAD may account for the partial dissociation of this enzyme in tissue extracts.

To achieve solubilization of the non-covalently bound dimers, immobilized hybrids were treated at 4 °C with 0.15 M NaCl, 5 mM EDTA, 2 mM dithiothreitol (pH 7.6).



Immobilized dimer of GAPD as an affinity sorbent. *A*, adsorption of dimers existing in the crude extracts of different tissues (a,b) with subsequent washing from unbound proteins. *B*, incubation under the dissociating conditions and washing of the sorbent, which may be used repeatedly. *C*, reactivation of the soluble dimers. The figures of different shape refer to various proteins.

A 30-min incubation under these conditions resulted in the splitting of 50% of the matrix-bound protein; the solubilized dimers were inactive (figure, B). Reactivation was accomplished under the conditions previously described to reverse cold inactivation of the soluble rat muscle enzyme[10]; the samples were incubated at 25 °C in the presence of 0.1 M sodium phosphate and 2 mM dithiothreitol for 15–20 min. Enzyme activity then appeared in solution; in the case of the rat muscle dehydrogenase it corresponded to 26 U/mg protein. This value is significantly lower than the sp. act. of the crystalline enzyme preparation (60–100 U/mg protein), which is probably due to the incomplete reactivation of the solubilized protein. The possibility of partial denaturation of the enzyme in the course of dissociation and reactivation (steps B and C) is the main shortcoming of the method. These steps may be omitted when the aim of the investigation is to study some properties of the isolated enzyme which are not altered in the hybrid molecule (for example, kinetic parameters of GAPD). Preliminary experiments of this type were carried out with hybrids of the carboxymethylated yeast dimers and muscle dimers extracted from different mammalian tissues.

The principal advantage of the present method is its applicability in cases where an enzyme is to be isolated from a small portion of material or from a diluted protein solution. Immobilized subunits can be prepared using an accessible oligomeric enzyme capable of forming hybrids with the enzymes to be isolated. This approach may help enzyme purification procedures based on highly specific protein-protein interactions.

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Role of lactone ring of aflatoxin B_1 in toxicity and mutagenicity

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Summary. Cleavage of the lactone ring of aflatoxin B_1 results in a nonfluorescent compound that has greatly reduced biological activity. Mutagenicity, as measured by the Ames test, is reduced 450-fold compared to that of B_1, and toxicity, as measured by the chick embryo test, is reduced 18-fold.

Aflatoxin B_1, a secondary metabolite of *Aspergillus parasiticus* and *A. flavus*, may function as a potent toxin, a carcinogen, a teratogen, and a mutagen[1]. All toxins in this family have a coumarin nucleus fused to a bifurano moiety and contain either a pentenone ring or a 6-membered lactone (figure).