EVIDENCE FOR THE ACTIVITY OF IMMOBILISED MONOMERS OF TRIOSE PHOSPHATE ISOMERASE

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Received September 28,1975

SUMMARY: Chicken muscle triose phosphate isomerase was immobilised by attachment to Sepharose 4B. The immobilised dimeric enzyme was dissociated with guanidinium chloride to yield bound monomeric triose phosphate isomerase. This regained activity on removal of the denaturant, showing that isolated monomers possess activity; the apparent K_m of the immobilised subunits was the same as that of the immobilised dimers. Under appropriate conditions, it was possible to rehybridise the immobilised monomers to native dimers, and also to form a hybrid dimer from the chicken muscle and rabbit muscle enzymes.

INTRODUCTION: In the study of the relationship between the quaternary structure of a multi-subunit enzyme and its activity, it is of interest to know whether the isolated subunits are enzymically active, or whether association is necessary for activity. One approach to this problem is to attach the enzyme to a solid support via a single subunit, and to remove the others so that the properties of the isolated subunit may be studied under conditions where reassociation is not possible (1,2,3). Here we describe the application of this method to chicken muscle triose phosphate isomerase (EC 5.3.1.1) which we attached to Sepharose 4B activated by cyanogen bromide.

Triose phosphate isomerase is composed of two identical subunits which are dissociated by guanidinium chloride solutions at concentrations greater than 0.7M (4,5). McVittie et al (5) showed that the dissociated polypeptide chains had also unfolded in these conditions. If the guanidinium concentration is lowered by dilution or dialysis, denatured triose phosphate isomerase regains its activity (4,6) although the percentage

recovery of activity is variable, particularly at low protein concentrations where it rarely exceeds 75% (4 and Fell and White, unpublished observations). The rate of recovery of activity from the denatured state is a second order process (4,6), suggesting that the main pathway to the native state involves a rate-limiting association of inactive subunits. The crystallographic structure of chicken muscle triose phosphate isomerase has been recently published (7), and although the enzyme-substrate complex has not yet been mapped, it is suggested that the active site on each subunit is in part bounded by a length of polypeptide chain from the other subunit.

MATERIALS AND METHODS: Chicken muscle triose phosphate isomerase was prepared by extracting macerated chicken breast muscle with 10mM Tris-HCl, pH 7.0, in 0.5mM EDTA and 0.2% 2-mercaptoethanol. The remaining steps were carried out according to the method described in (5). The preparation gave a single band in gel electrophoresis in tris-glycine, pH 8.5, and had a specific activity of 7,200 $U.mg^{-1}$. All other enzymes were purchased from Boehringer (London).

The enzymic activity of both soluble and immobilised triose phosphate isomerase was measured at 25° by the assay method described in (5). α-glycerophosphate dehydrogenase (EC 1.1.1.8), the secondary enzyme in the assay, was dialysed against 50% glycerol/50% 0.1 M triethanolamine, pH 7.4, plus 1% 2-mercaptoethanol to enhance its stability and to remove contaminating sulphate ions (8).

The method of Porath et al (9) was followed for the immobilisation of the enzyme. A number of samples of Sepharose 4B were activated with different concentrations of cyanogen bromide, were washed with 0.5 M $NaHCO_3$ and 0.1 M phosphate, pH 8.0, and stirred overnight at 4°C together with a large excess (up to 5mg) of soluble enzyme. The gel bearing the immobilised enzyme was then washed with 0.1 M triethanolamine-HCl, pH 7.4, until no further activity was eluted.

The denaturation of immobilised triose phosphate isomerase was carried out at room temperature by eluting a column of gel with 2 M guanidinium chloride (Sigma); denaturation is complete in less than 5 minutes under these conditions (6). Renaturation of the enzyme was accomplished by washing a sample of the gel from the column with 0.1 M triethanolamine-HCl, pH 7.4, 100mM 2-mercaptoethanol.

Rehybridisation studies of the bound monomeric triose phosphate isomerase formed by denaturation were carried out by passing a linear gradient (2 - 0 M guanidinium chloride in 0.1 M triethanolamine-HCl, pH 7.4, 100mM 2-mercatoethanol) containing 20 Units of enzyme per ml of gel through a packed column, and washing with renaturing buffer until no further activity was eluted.

RESULTS AND DISCUSSION: In the preparation of triose phosphate isomerase bound to Sepharose, we found that there was a linear relationship between the amount of cyanogen bromide used to activate the gel and the enzymic activity of the product within any one experiment (Fig. 1). The amounts of cyanogen bromide used in these experiments between 0.5 and 5 mg per ml of packed gel; at the upper end of this range, Chan and Mawer (1)

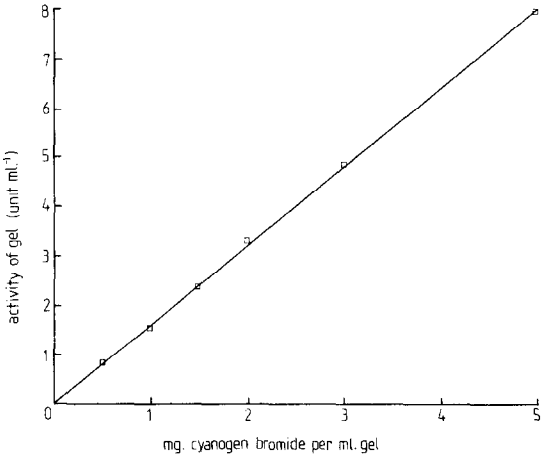

Figure 1 - Immobilised triose phosphate isomerase activity at varying degrees of cyanogen bromide activation.

found evidence for some degree of multi-point attachment of aldolase; however, it is clear from their results that the amount of activity immobilised per unit weight of cyanogen bromide was decreasing as the amount of cyanogen bromide was increased; further, the greater efficiency of their coupling conditions resulted in the amount of protein which they immobilised (of the order of 100 $\mu g.ml^{-1}$) being much greater than the amount of triose phosphate isomerase which we coupled to the gel (below the sensitivity of protein assay methods, but probably of the order of 0.1 - 10 $\mu g.ml^{-1}$, estimated from the specific activity of the enzyme). Nagradova et al (3) reported no evidence of multi-site attachment of glyceraldehyde-3-phosphate dehydrogenase although they used 20 mg of cyanogen bromide per ml of Sepharose.

The kinetic properties of triose phosphate isomerase are altered by immobilisation on Sepharose; the apparent K_m of immobilised enzyme for glyceraldehyde-3-phosphate, measured at the optimal conditions for the soluble enzyme, was 1.7mM, which is significantly higher than our value

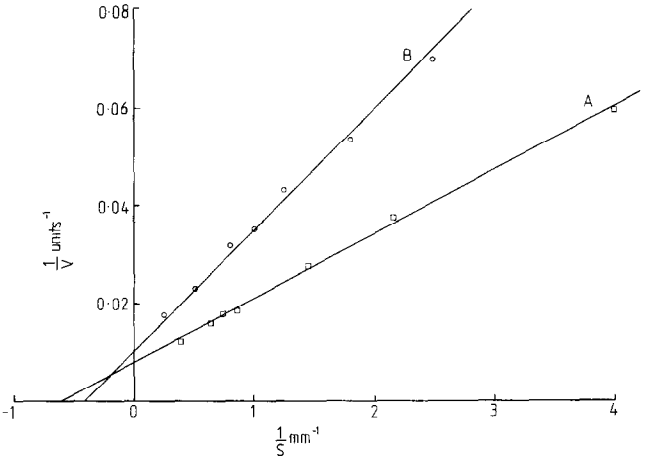

Figure 2 - Lineweaver-Burk plot showing the dependence of immobilised triose phosphate isomerase activity on concentrations of glyceraldehyde-3-phosphate.

A - Native enzyme
B - Denatured/renatured enzyme.

of 0.35mM for the K_m of the soluble enzyme (cf. 0.47mM, 10).

When a column of immobilised triose phosphate isomerase was eluted with 2 M guanidinium chloride, enzymic activity was detected in the eluate at the solvent interface; although the eluted enzyme was denatured, it underwent renaturation in the assay cuvette. However, it was not possible to estimate the total amount of activity eluted from the gel because the degree of renaturation was unknown. Samples of gel removed from the column and washed with renaturing buffer showed recovery of activity in all cases, although the percentage of the original activity regained was again variable, between 20% and 60%. The renatured activity, which represents that of the bound monomers, has an apparent K_m of 2mM for glyceraldehyde-3-phosphate, similar to that of the immobilised native dimers (Fig. 2).

The remainder of the gel in the column was washed with a decreasing linear gradient of guanidinium chloride in the presence of soluble triose phosphate isomerase. After washing the column with buffer until the eluate was free of enzymic activity, the gel was assayed to detect whether the bound monomers had rehybridised with monomers in solution to form bound dimers. In eight experiments, the activity of the rehybridised enzyme was 1.8 (± 0.25) times higher than the activity recovered by the renatured samples from the same column. A control experiment in which the gradient was run without the soluble enzyme present showed that the gradient method of renaturation gave the same activity as the direct washing of denatured gel with renaturing buffer. This confirms that the guanidinium chloride has depleted the enzyme gel of subunits, and that these can be replaced under suitable rehybridising conditions. That the activity of the gel renatured under rehybridising conditions is close to twice that of the gel renatured in the absence of free enzyme monomers suggests that the specific activities of the monomers and dimers are similar.

In two experiments, the denatured gel was rehybridised with soluble rabbit muscle triose phosphate isomerase; in both cases, an increase in

activity compared with renatured gel enzyme (2.8 and 1.5 times higher) was observed. We conclude that it is possible to form active hybrids of the chicken and rabbit muscle enzymes, which demonstrates that the 32 differences in amino acid sequence between these two enzymes (11) has not significantly altered the subunit interface region.

ACKNOWLEDGEMENTS: We would like to thank Drs. J.D. McVittie and M.P. Esnouf for helpful discussions in the early stages of this work.

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