EVIDENCE FOR ACTIVE SUBUNITS OF MATRIX-BOUND CREATINE KINASE

Gordon F. BICKERSTAFF and Nicholas C. PRICE
Department of Biochemistry, University of Stirling Stirling FK9 4LA, UK

Received 27 February 1976

1. Introduction

Creatine kinase (EC 2.7.3.2.) which catalyses the transfer of a phosphoryl group between ATP and creatine consists of two very similar, if not identical, subunits of mol wt. 41 000, each possessing a highly reactive thiol group [1]. The dimeric enzyme is readily dissociated in denaturing media such as 6 M guanidinium hydrochloride (Gdn HCl) or 8 M urea [2]. In considering the role of the subunits of the enzyme it is of value to determine whether or not individual subunits can show enzymatic activity. There have been two principal approaches to the study of the activity of subunits of oligomeric enzymes. The first approach involves an analysis of the kinetics of regain of activity during re-association of the dissociated enzyme [3]. The second approach involves preparation of matrix-bound subunits which are unable to re-associate upon removal of the denaturing agent. This latter method was used successfully by Chan to study isolated subunits of aldolase [4] and transaldolase [5] and was adopted here for the study of creatine kinase. In the present communication we outline a method for attaching rabbit muscle creatine kinase to Sepharose and present evidence to show that individual subunits of the matrix-bound enzyme are active.

2. Materials and methods

Creatine kinase was isolated from rabbit skeletal muscle as described by Milner-White and Watts [6]. Freshly prepared enzyme had a specific activity (assayed in the forward direction ATP + creatine → ADP + phosphocreatine) of 55–60 units/mg under the conditions described by McLaughlin et al. [7] and 130–140 units/mg under the conditions described by Milner-White and Watts [6]. The enzyme preparations were judged to be more than 90% homogeneous by sodium dodecyl sulphate polyacrylamide gel electrophoresis [8].

Sepharose 4B (Pharmacia) was activated according to the procedure of March et al. [9] but using 5 mg CNBr per ml of packed gel. After activation, the gel was washed successively with 0.1 M sodium bicarbonate at pH 9.0, distilled water, and finally the coupling buffer (10 mM sodium phosphate at pH 8.0 containing 1 mM EDTA). To 10 ml of activated gel was added 10 mg of enzyme in 8 ml of coupling buffer, and the mixture was stirred at 4°C for 18 h. Excess soluble protein was then removed by washing the gel alternately with coupling buffer containing 1 M NaCl and coupling buffer containing no NaCl, until no protein could be detected in the washings. A volume of 0.1 M sodium glycine at pH 8.0, equal to the volume of packed gel was then added to the matrix-bound derivative and the mixture was left to stand at 20°C for 2 h to allow the complete blocking of remaining activated groups on the Sepharose. After washing to remove excess glycine, the gel was suspended in coupling buffer. Dissociation of the matrix-bound enzyme by Gdn HCl and subsequent re-association with added subunits of soluble enzyme were performed essentially according to the procedures described by Chan [4].

The concentration of soluble enzyme was determined either spectrophotometrically at 280 nm using the published value for the extinction coefficient [6], or by the method of Lowry et al. [10] using bovine serum albumin as a standard. The two procedures gave identical results. Protein concentrations of matrix-bound derivatives were determined using a slightly modified version of the Lowry method used by Havekes

et al. [11]. The mixture of matrix-bound enzyme and alkaline copper tartrate reagent was stirred for 10 min prior to addition of the Folin-Ciocalteau reagent. After the addition and a further 30 min stirring, the mixture was filtered before the absorbance was determined. With this procedure, protein determinations were highly reproducible and the absorbance was linear with protein content at least up to 45 μg of protein.

Creatine kinase activity was assayed using the coupled assay system described by McLaughlin et al. [7]. The activity of matrix-bound derivatives was determined by addition of a small aliquot (10–100 μl) of suitably diluted suspension to the assay mixture, which was maintained at 25°C and continuously stirred using an apparatus described by Mort et al. [12] At suitable (2 min) intervals the cuvette containing the mixture was placed in a spectrophotometer to record the absorbance at 340 nm for a few seconds. Under these conditions the observed activity was proportional to the amount of enzyme added to the assay mixture (0.2–0.8 μg enzyme). The contribution of any remaining soluble enzyme to the observed activity was checked by filtration of the mixture and found to be less than 2% in all cases. Pipetting of gel suspensions was found to be most accurately and conveniently carried out using a variable automatic pipette with the plastic tips cut so as to increase the size of the aperture.

3. Results and discussion

As shown in table 1 creatine kinase could be linked to Sepharose 4B with retention of approx. 50% of the specific activity of the soluble enzyme. This figure is comparable with the results of other studies on matrix-bound enzymes [4,5,13,14] and could possibly arise either from distortion of the enzyme or a change in the microenvironment of the catalytic site upon immobilisation. Further investigation of the kinetic and binding properties of the matrix-bound enzyme may help to clarify this point.

The preparation of matrix-bound subunits of creatine kinase involves treatment of the matrix-bound enzyme with a denaturing agent, followed by washing to remove dissociated subunits. The ability of soluble creatine kinase to renature upon removal of denaturing agent was checked under conditions similar to those used in the study of matrix-bound derivatives. A sample of enzyme which had been incubated at a concentration of 0.6 mg/ml in denaturing buffer (0.1 M Tris-HCl at pH 7.5 containing 12.5 mM dithiothreitol and 6 M Gdn HCl) for 30 min at 20°C was diluted 10-fold into renaturing buffer (0.1 M Tris-HCl at pH 7.5 containing 12.5 mM dithiothreitol). At known times, aliquots were then removed for assay. As shown in fig.1 about 80–85% of the original activity of the enzyme before treatment with Gdn HCl could be recovered after 1 h. These results were comparable with those previously obtained [2] under slightly different conditions.

Extensive washing of matrix-bound enzyme (derivative A) with the above denaturing buffer yielded a derivative which had very nearly half the protein content of derivative A (table 1). A control experiment showed that treatment with the same buffer without Gdn HCl had no effect on the protein content or activity of derivative A. These results show that the enzyme is bound to the matrix almost exclusively

Table 1
Activity and protein content of matrix-bound creatine kinase derivatives

Matrix-bound Derivative	Protein content μg/ml	%	Activity U/ml	%	Specific activity U/mg	%
Derivative A	400	100	11.2	100	28	100
Derivative B	210	52.5	5.4	48	25.7	92
Derivative C	380	95	10.4	93	27.4	98

Derivative A was prepared by coupling creatine kinase to Sepharose 4B. Derivative B was prepared by washing Derivative A with denaturing buffer (containing guanidinium hydrochloride). Derivative C was prepared by adding dissociated soluble enzyme to Derivative B. The accuracy of protein content and activity determinations was ± 5%. Soluble creatine kinase had a specific activity of 55–60 U/mg.

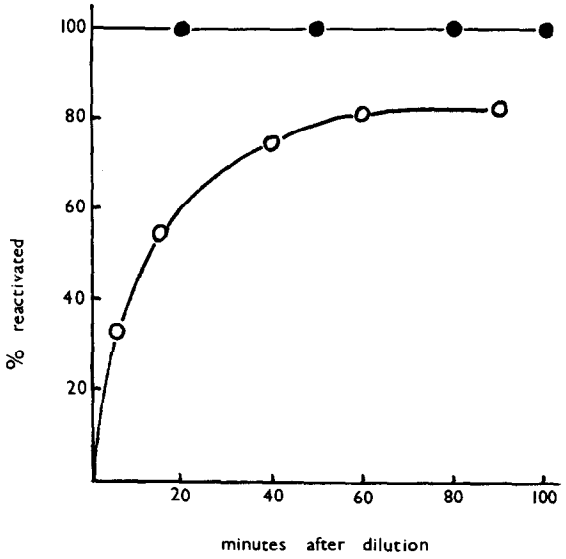

Fig.1. Reactivation of soluble creatine kinase from 6 M guanidine-HCl, in 0.1 M Tris-HCl at pH 7.5 containing 12.5 mM dithiothreitol. At zero time the denatured enzyme solution was diluted 10-fold into the above buffer without guanidine-HCl and assayed at the stated times (○). A parallel control experiment in which the enzyme was incubated in 0.1 M Tris-HCl at pH 7.5 containing 12.5 mM dithiothreitol showed no loss of activity (●).

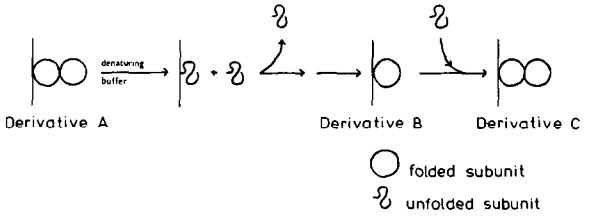

Fig.2. Scheme demonstrating the relationship between the derivatives of matrix-bound creatine kinase. Derivative A represents matrix-bound enzyme. Successive treatment of Derivative A with denaturing and renaturing buffer yielded Derivative B. Derivative C was derived from Derivative B by re-association with 'added' subunits of soluble enzyme.

via one rather than via both subunits (since in the latter case there would be no protein loss from derivative A after treatment with Gdn HCl). Following the washing of derivative A with denaturing buffer, the gel was suspended in the renaturing buffer, stirred for 1 h at 20°C, washed with coupling buffer and finally suspended in coupling buffer. The resulting product (derivative B) was found to have a specific activity of 92% compared with derivative A (Table). These data would suggest that matrix-bound subunits of the enzyme are active.

Stronger evidence for the presence of matrix-bound subunits in derivative B was provided by its ability to re-associate with added 'subunits' of soluble creatine kinase (see scheme in fig.2). In this procedure small aliquots of a solution (2 mg/ml) of soluble enzyme (which had been incubated in the denaturing buffer for 1 h at 20°C) were added to a well stirred suspension of derivative B in renaturing buffer (1:4). An excess (1 mg) of the dissociated soluble enzyme was added in this way over a period of 90 min. After the addition, the mixture was stirred for a further 2 h at room temperature to allow renaturation to occur. The product was then washed and re-suspended in coupling buffer, to yield derivative C (table 1). The results of the experiment show that the protein content and activity are restored to very nearly the original values of derivative A. Control experiments showed that neither derivative A nor washed non-activated Sepharose 4B could retain added subunits under the same conditions.

Taken together, the results shown in table 1 indicate that matrix-bound subunits of creatine kinase can be prepared and that these subunits possess a specific activity very similar to that of matrix-bound enzyme. The observation of active subunits of creatine kinase is of particular interest since the related enzyme arginine kinase occurs in a monomeric form in a number of species, particularly lobster [15]. Previous work has indicated that lobster arginine kinase bears a notable similarity to the creatine kinase subunit in terms of molecular weight and amino acid sequence around the rapidly reacting thiol group [15]. We plan to compare the properties of matrix-bound creatine kinase in the dimeric form and in the subunit form to assess the importance of the dimeric structure of the soluble enzyme.

Acknowledgement

We thank the Science Research Council for a studentship (G.F.B.) and for general financial support.

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