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An example of substrate channeling between co-immobilized enzymes

Coupled activity of myosin ATPase and creatine kinase bound to frog heart myofilaments

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In myofilaments obtained by Triton X-100 lysis of frog heart cells in high ionic strength medium, the activity of bound creatine kinase cannot be detected by a coupled enzymatic assay. ATP is channelized toward myosin ATPase, through the unstirred layer near myofilaments and cannot diffuse into the bulk solution. Model systems based upon the coupled kinetics of enzymes co-immobilized on the same surface may explain this behaviour. This may also account for why myofilament-bound creatine kinase is more efficient than free enzyme in the cytosol for the physiological recycling of ADP into ATP.

Substrate channeling; Immobilized enzyme; Creatine kinase; Myofilament; (Frog heart)

1. INTRODUCTION

Kinetic properties of the isolated enzymes are generally well documented on purified proteins. The problem is to extrapolate these in vitro properties to the cellular behaviour of these enzymes. There is growing evidence that proteins associate with each other and with various membranous and particulate components of the cell. The binding of enzymes of a pathway on the same cellular matrix may promote substrate channeling in the unstirred layer (or Nernst layer) near the surface. Model systems of enzymes co-immobilized on a surface [1,2] show this channeling, but there are few examples at the cellular level [3]. Creatine kinase (EC 2.7.3.2 or CK) in muscle and heart cells is a good example of such an immobilization. This enzyme catalyzes the phosphate transfer between creatine phosphate and ADP:

$$\text{MgADP} + \text{creatine phosphate} + \text{H}^+ \rightleftharpoons \text{MgATP} + \text{creatine}$$

with an equilibrium constant of $10^9\ \text{M}^{-1}$ in the presence of 1 mM free Mg [4]. CK is found as four isoenzymes: three cytosolic forms MM, MB and BB, named according to the tissue where they are predominantly found (MM in muscle, BB in brain), and a mitochondrial form, bound to the external face of the internal membrane [5]. A part of the MM form is firmly bound to the M line of the myofilaments [6] and can only be detached in low ionic strength medium [7].

In the present study, evidence is shown of ATP channeling between CK and myosine ATPase in Triton-extracted frog heart cells. The model system previously described for aspartate aminotransferase and malate dehydrogenase co-immobilized on collagen films [2] may explain this

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channeling in the boundary layer near myofilaments.

2. MATERIALS AND METHODS

2.1. *Kinetic model*

The coupled activity of two enzymes E_1 and E_2 can be represented by the following reaction:

$$S \xrightarrow{E_1} P1 \xrightarrow{E_2} P2$$

In the case of an enzyme bound to a surface, the enzymatic reaction takes place at the surface and the substrate has to flow from the bulk solution to the active surface through the so-called 'unstirred layer'.

The kinetic equations had been developed for the enzymes either free in solution or bound to a support, assuming simple first order kinetics for both E_1 and E_2 [1,2]. Here these equations were extended to the case where either bound or free E_1 is acting at its maximum velocity because the concentration of the substrate S is very high, and where E_2 obeys first order kinetics.

Defintions: h, transport coefficient of a substrate or a product between the enzymatic surface and the bulk solution, $h = D/l$, where l is the thickness of the boundary layer and D is the translation diffusion coefficient (almost the same for substrates and co-enzymes); v, volume of the substrate solution; a, surface of the enzymatic film; k_2, first order kinetic constant for the second enzyme in solution; V, maximum velocity of the first enzyme; P_1 and P_2, concentrations of the first and second product in the bulk solution.

(i) For E_1 alone, either in solution or bound to a surface, $P_1 = V \cdot t$. For coupled enzymes in all cases, $P_2 = V \cdot t - P_1$.

(ii) Coupled kinetics of E_1 and E_2 free in solution:

$$P_1 = (V/k_2) \cdot [1 - \exp(-k_2 \cdot t)]$$

(iii) Both enzymes immobilized on the same surface:

$$P_1 = (V/k_2) \cdot (1 - \exp[-k_2 \cdot h \cdot t/(\frac{v}{a} \cdot k_2 + h)])$$

$$\approx V \cdot h \cdot t/(\frac{v}{a} \cdot k_2 + h), \text{ when } k_2 \cdot h \cdot t/(\frac{v}{a} \cdot k_2 + h) < 0.15$$

(iv) First enzyme in solution, second enzyme bound to a surface:

$$P_1 = [V \cdot (\frac{v}{a} \cdot k_2 + h)/k_2 \cdot h] \cdot (1 - \exp[-k_2 \cdot h \cdot t/(\frac{v}{a} \cdot k_2 + h)])$$

$$\approx V \cdot t, \text{ when } k_2 \cdot h \cdot t/(\frac{v}{a} \cdot k_2 + h) < 0.15$$

2.2. *Myofilaments obtained by Triton treatment of isolated ventricular cells from frog*

Cells were isolated as previously described [8]. The isolated cells were mixed for 15 s at 2°C with lysis medium containing 2% Triton X-100, 3 mM EDTA, 20 mM morpholinopropane sulfonic acid, 115 mM KCl, pH 7.1, instead of 6.75 used in previous work [9,10] and the myofilaments were obtained as a pellet after centrifugation through silicone oil. The pellet was resuspended either in the above medium without Triton, or in the buffer used for ATPase activity determination. The CK activity was determined in the pellets and in the supernates of silicone oil centrifugation. The total cellular CK activity was determined on cells treated by Triton X-100 in medium of low ionic strength (lysis medium plus 230 mM mannitol instead of KCl). The myofilament concentration was expressed relative to the protein content of the initial cell suspension.

2.3. *ATPase activity*

The myosin ATPase activity was determined in 50 mM triethanolamine buffer, pH 7.2, containing 75 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, 50 μM phospho*enol*pyruvate, 4 mM ATP, 10 μM NADH, 1.25 IU/ml pyruvate kinase and 1.25 IU·ml lactate dehydrogenase. The reaction was started by adding an aliquot of myofilament suspension and the decrease of NADH fluorescence was studied by fluorometry in a JY3D fluorometer (excitation, 340 nm; emission, 460 nm). The response was calibrated by adding a known amount of NADH to the cuvette. The activation by calcium was studied by addition of 0.4 mM $CaCl_2$ and the absence of contaminant ATPase activities was checked by adding either 5 mM NaN_3 or 10 μM ouabain.

2.4. *Creatine kinase*

This activity was determined in 50 mM Tris-acetate buffer, pH 7.2, containing 4.2 mM $MgSO_4$, 2.1 mM K form ADP, 6.7 mM glucose, 0.6 mM NADP, 1.75 IU/ml glucose 6P dehydrogenase, 1 IU/ml hexokinase and an aliquot of myofilament suspension. The K form of ADP was used because it contains neither ATP nor AMP. After a 10 min incubation to determine contamination by myokinase activity ($2ADP \rightleftharpoons ATP + AMP$), the reaction was started by addition of 3.3 mM creatine phosphate. As previously observed [10], the total cellular activity of myokinase is lower than 5% of the total CK activity and myofilaments prepared by Triton lysis are devoid of myokinase activity.

2.5. *Materials*

Enzymes were from Boehringer, Triton X-100, NADP, NADH, ATP and ADP from Sigma and silicone oil from Serva.

3. RESULTS AND DISCUSSION

3.1. *ATPase activity*

An example of the ATPase activity of myofilaments is shown in fig.1. This activity is increased by calcium and insensitive to either ouabain or NaN_3, i.e. not contaminated by Na^+,K^+-ATPase or mitochondrial ATP synthase. The activity was 25 ± 5 nmol ATP/min per mg initial cellular protein, without added calcium (four cellular isolations, duplicate determinations). According to the myosin content of ventricular cells [11], it corresponds approximately to a $k_{cat} = 1\ s^{-1}$ for myosin ATPase in frog heart myofilaments. A 2–3

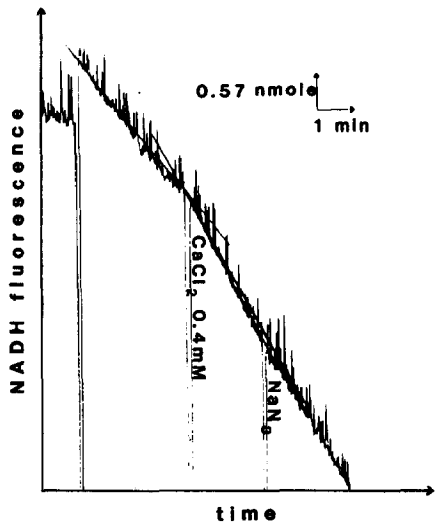

Fig.1. ATPase activity of myofilaments obtained from Triton-extracted frog heart cells (25 μg total cellular protein). The activity is activated by calcium and insensitive to NaN_3.

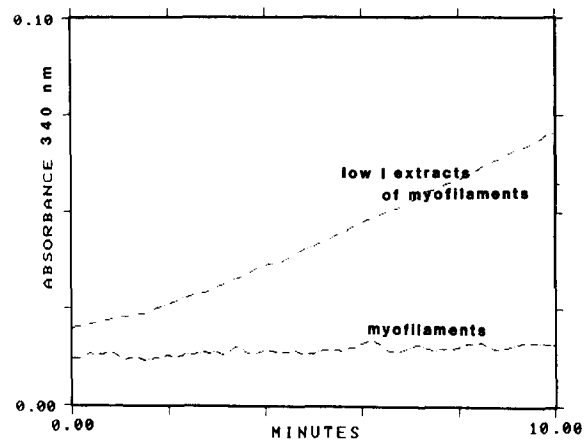

Fig.2. Creatine kinase activity (detection of formed ATP) of frog heart myofilament (75 μg total cellular protein) prepared in high *I* medium, observed before and after treatment with a medium of low *I*.

times increase of the ATPase activity was observed in the presence of calcium.

3.2. *Creatine kinase activity*

In previous experiments [9,10], the myofilaments had been prepared by lysis of isolated frog heart cells in high ionic strength medium at pH 6.75. After centrifugation, through silicone oil, of the myofilaments prepared at this pH, 80–75% of the total CK activity was obtained in the supernates; nevertheless, a treatment of the pellet with a low ionic strength medium was necessary to obtain 20–25% activity in the myofilament fraction [10].

In the present work, the Triton lysis has been performed at an higher pH, 7.1 instead of 6.75. Under these conditions, 90% of the total CK activity was obtained in the supernates of the silicone oil centrifugation. It is very likely that some loosely bound CK [12] became detached from the myofilaments at this higher pH. In the bottom myofilament fraction, no CK activity was apparent by means of the ATP detection in the coupled enzymatic assay (fig.2). Nevertheless, firmly bound creatine kinase was present in this fraction since it may be obtained after treatment by a low ionic strength medium (fig.2). The solubilized CK activity was 12.5 ± 2 nmol ATP/min per mg initial cell protein (four cell isolations, duplicate determinations). In no case was myokinase activity observed in these myofilaments.

The CK activity was studied in the present work, by determination of ATP produced from ADP and creatine phosphate, by means of a coupled enzymatic assay using hexokinase and G6P-dehydrogenase. In this assay competition between hexokinase and myosin ATPase is possible, but the 1000 times excess of hexokinase over myosin ATPase activity should render this competition negligible in solution: this is what is observed when CK is solubilized. On the other hand, when CK is bound to the myofilaments, it is very likely that ATP coming from the CK reaction is hydrolyzed by myosin ATPase before diffusing into the bulk solution, so that hexokinase cannot use ATP (fig.3).

The simplified kinetic model described for two enzymes co-immobilized on a surface may explain this behaviour. E_1 is CK, and the concentration of ADP and creatine phosphate are high enough to obtain the maximum velocity of CK (V = 0.87 $\mu M \cdot min^{-1}$). E_2 is myosin ATPase, and the low rate of ATP formation gives a plausible first order kinetic for this enzyme. The first order kinetic constant $k_2 = V_m^{ATPase}/K_m^{ATPase}$ may be estimated to be 0.09 min^{-1} for the myofilaments used in fig.2 (V_m^{ATPase} = 1.3 $\mu M \cdot min^{-1}$; K_m^{ATPase} = 14 μM [13]). v/a = 10 cm (v = 2 ml, volume of the spec-

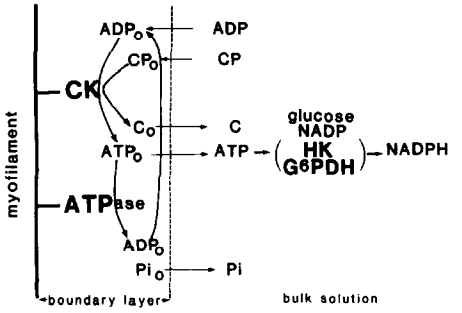

Fig.3. Scheme of the coupled activity of myosin ATPase and creatine kinase (CK) bound to myofilaments (HK, hexokinase; G6PDH, glucose-6-P dehydrogenase; C, creatine; CP, creatine phosphate).

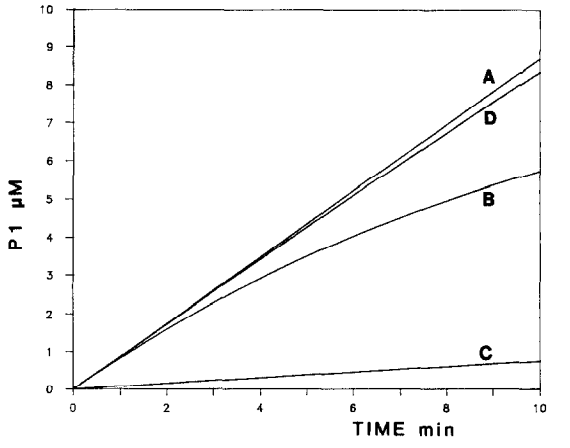

Fig.4. Simulation of the time dependence of the appearance in the bulk solution of the intermediate P1 of the coupled kinetics of two enzymes E_1 and E_2. (A) Only E_1 (free or bound to a surface); (B) both enzymes free in solution; (C) both enzymes immobilized on the same surface; (D) E_1 free in solution, E_2 immobilized on a surface. Curves C and D correspond to the experimental curves shown in fig.2.

trophotometer cuvette; $a = 0.2\ cm^2$, corresponding to the external surface of the 7000 treated cells in suspension in the cuvette). P_1 is the concentration of ATP in the bulk solution, i.e. the ATP detectable by hexokinase in the coupled enzymatic assay. The transport coefficient h is $0.09\ cm \cdot min^{-1}$ for MgATP [2]. In fig.4 the time course of the concentration P_1 in the bulk solution is simulated. During the first few minutes, the accumulation of intermediate P1 in the bulk solution (i.e. ATPase titratable by the coupled enzymatic assay) is high for enzymes free in solution (curve B) and still higher for a free enzyme coupled to a bound one (D). This last case corresponds to CK liberated by low ionic strength medium, in the presence of myosin ATPase: the competition between the 1000 times excess of hexokinase and myosin ATPase is negligible. When both enzymes are immobilized on the same support, almost no intermediate is observed in the bulk solution (curve C) because this intermediate formed in the boundary layer is used by E_2 before diffusing into the medium. This is observed when CK is bound to myofilaments.

These simulations indicate that the observed channeling of ATP between CK bound to frog heart myofilaments and myosin ATPase may be explained by transfer of the metabolite in the unstirred layer around the myofilaments, without hypothesis of an intimate coupling between the enzymes [14].

The model gives a description of the effect of immobilization of CK on the facilitated diffusion of ATP in isolated myofilaments. In this system, the limitation by external diffusion is important, due to an unstirred layer of about 10 µm. As in heart cells the distance between the source of ATP, mitochondria, and myofibrils is far lower than the unstirred layer, external diffusion is of less importance in vivo than in solution. In vivo, the description of the facilitated diffusion of ATP and ADP by creatine phosphate and creatine [15] has to take into account that CK is bound near sites of ATP production and utilization; this is possible with very sophisticated models [16].

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