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## A model of mitochondrial creatine kinase binding to membranes: adsorption constants, essential amino acids and the effect of ionic strength

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The quantitative aspects of mitochondrial creatine kinase (mitCK) binding to mitochondrial membranes were investigated. A simple adsorption and binding model was used for data fitting, taking into account the influence of protein concentration, pH, ionic strength and substrate concentration on the enzyme adsorption. An analysis of our own data as well as of the data from the literature is consistent with the adsorption site of the octameric mitCK being composed of 4 amino acid residues with  $pK = 8.8$  in the free enzyme. The  $pK$  value changes to 9.8 upon binding of the protein to the membrane. Lysine is suggested as the main candidate to form the adsorption site of mitCK. Deprotonated octameric mitCK easily dissociated from the membrane ( $K_a = 0.39$  mM at ionic strength  $I = 7.5$  mM and  $5^\circ\text{C}$ ); after protonation its affinity increased many times ( $K_{ah} = 39$  nM). Determination of mitCK adsorption capacity by another method at pH 7.4, when the enzyme is almost protonated, gave  $K_{ah} = 15$  nM. The effect of ionic strength on mitCK adsorption may be described in terms of Debye-Hückel's theory for activity coefficients assuming the charges of the interacting species to be +4 and -4. The dissociation constant for the mitCK-membrane complex at pH 7.4 and  $I = 0$  was evaluated by different approaches as approx. 1 nM. Extramitochondrial ATP (or ADP) shifted greatly the equilibrium between the adsorbed and the free mitCK towards the solubilized state, since in the adsorbed protein the external ligands had access to four binding sites and in the free protein to eight sites.

### Introduction

Mitochondrial creatine kinase (mitCK) is a crucial enzyme at the beginning of the creatine-creatine phosphate (Cr-CrP) energetic circuit in muscle, brain and other tissues [1,2]. The existence of this circuit is due to a preferential supply to mitCK, situated between the inner and outer membranes, of ATP synthesized in the mitochondrion. Several hypotheses have been proposed to explain the preference of mitCK to intra mitochondrial ATP. One of them claims a structural and functional coupling between adenine translocase, executing ATP-ADP exchange through the inner membrane, and mitCK [3,4]. This coupling provides a direct passage of ATP between the active sites. Another explanation [5,6] stresses the role of the mitochondrial outer membrane for ATP compartmentation inside the mitCK pool. Recently a third hypothesis was

developed [2]. It assumes mitCK to be a modulator of contacts between the inner and outer membranes. Direct substrate tunnelling takes place inside these contacts.

All these models require a knowledge of the mechanism of mitCK binding to the mitochondrial membrane. However, so far the data on the subject are qualitative rather than quantitative. It is known that at neutral pH, positively charged mitCK binds electrostatically to the inner membrane of mitochondria [7–11]. Binding to negatively charged cardiolipin is more probable than to positively charged translocase [11–13]. After disruption of the outer mitochondrial membrane mitCK may easily be solubilized by different compounds which increase the ionic strength (KCl, NaCl,  $P_i$  etc.) [7–10]. Certain ligands (ATP and ADP but not Cr or CrP) exhibit more pronounced solubilizing properties [8–10].

There are contradictory data about which amino acids are essential for mitCK adsorption. Thus, mercuriate modification of essential SH-groups leads to mitCK extraction from mitoplast [12,14]. However, other SH-reagents do not affect mitCK binding [12,14].

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Abbreviations: mitCK, mitochondrial creatine kinase; Cr, creatine; CrP, creatine phosphate.

Chemical modification of the positively charged histidine, arginine and lysine residues prevents adsorption of 21%, 91% and 94% of the enzyme, respectively [12]. The crucial role of Arg-19 and Lys-20, having side chains with high alkaline  $pK$ , was claimed by Cheneval and Carafoli [12]. In contrast, solubilization of mitCK by increasing pH usually took place at neutral or slightly alkaline pH, with half-solubilizing pH: 6.8 [7], 7.3 [10] and 8.4 [15]. Lipskaya et al. [9] noted no solubilization at pH 6–9.

MitCK binding to the membrane is not only of an electrostatic nature, since 88% of it stays bound to the membrane under certain conditions even at high ionic strength [16]. It is well known that 10–30% of tightly bound mitCK activity remains on the membrane even after a series of solubilizations at high ionic strength [2]. The existence of two oligomeric forms of mitCK, octameric ( $M_r = 340$ ) and dimeric ( $M_r = 84$ ), with different affinities towards the membrane, and capable of inter conversion at certain conditions, also complicates the process [2].

In this paper we present an analysis of our own data as well as data from the literature on mitCK binding to the membrane. We consider the effects of enzyme concentration, pH and ionic strength.

## Materials and Methods

Creatine kinase was isolated from beef heart mitochondria as described earlier [17,18]. The enzyme was stored in 10 mM  $P_i$ -buffer containing 0.2 mM dithiothreitol at 5°C.

The specific activity of the enzyme was determined pH-metrically in the reaction  $CrP^{2-} + MgADP^{1-} + H^+ \rightarrow Cr + MgATP^{2-}$ . The incubation medium contained: 10 mM Tris-HCl, 10 mM KCl, 6 mM magnesium acetate, 10 mM CrP, 1 mM ADP (pH 7.4), 30°C. The activity of the purified mitCK was 120–150 IU/mg.

The protein concentration was determined by spectroscopy using the absorbing index  $A_{280}^{1\%} = 9.0$  at  $A_{280}/A_{260} = 1.4$ –1.5 or a nomogramme at  $A_{280}/A_{260} < 1.4$ . Comparison with the data obtained by the microbiuret method using creatine kinase from rabbit muscle as a standard revealed an error of less than 10% in the former method. The total concentration of protein in the mitochondria was determined by the microbiuret method.

Non-equilibrium ultracentrifugation of the enzyme was performed on a Beckman E Spinco centrifuge.

MitCK-free mitochondria for binding experiments were obtained during the process of mitCK purification by swelling in water and salt extraction of the mitCK. The mitCK-activity of swelled mitochondria was 1.1–1.3 IU per 1 mg of mitochondrial protein. After salt treatment the membranes contained 0.1–0.3 IU/mg of tightly bound mitCK.

MitCK adsorption on the mitochondrial membranes was carried out in 5 mM Tris, 5 mM glycine buffer at varying pH in the presence of 1 mM dithiothreitol. Before the experiment the mitochondria, stored in 0.25 M sucrose, were prewashed with the buffer.

0.1 ml of mitCK (1–2 mg/ml) in 0.01 M  $P_i$ -buffer (pH 7.4) was added to mitochondria (2.5 mg/ml or 25 mg/ml) suspended in 1 ml Tris-glycine buffer. After incubation for 10 min at 5°C with constant stirring, the mitochondria were separated by centrifugation for 10 min at  $22000 \times g$ , 5°C, and suspended in 1 ml 0.05 M  $P_i$ -buffer (pH 7.4) to release mitCK for the catalytic activity assay. 0.15 ml of 0.3 M  $P_i$ -buffer (pH 7.4) was added to 0.9 ml of supernatant to increase the ionic strength and normalize the pH. The amount of mitCK originally bound to the membrane was evaluated by measurement of the activity of the thus solubilized enzyme. The net gain of the activity after rebinding corresponded to the quantity of newly adsorbed mitCK. The increase in the enzyme activity in the pellet was equal to its decrease in the supernatant. The specific activity of mitCK tightly bound to mitochondria was either preliminarily suppressed by 5 mM iodoacetamide (for 1 min) or its value was subtracted from the total activity. These two methods gave identical results.

Data fitting was done using a nonlinear regression computer program (Fedosov, S.N., unpublished) installed on an IBM AT computer.

## Theory

In general, adsorption may be described by the model presented in Fig. 1A. To evaluate the parameters of this model the dependence of adsorption on  $[E]$  and pH should be analyzed. Also, the influence of ionic strength has to be taken into account.

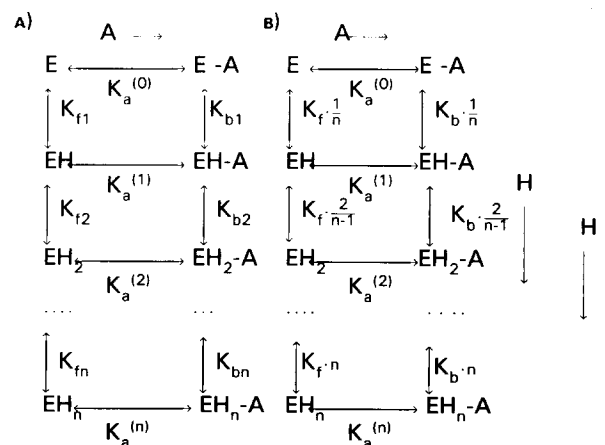
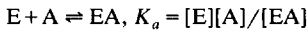


Fig. 1. The simple model for binding of the ligand H to the enzyme E and interaction of E with the adsorbent A, see text. (A) General scheme; (B) Scheme proposed for mitCK. All  $K_i$  are dissociation constants.

### Binding equilibria

Consider first the simple binding equilibrium between enzyme E and adsorbent A (in our case mitochondrial membranes):



If the concentrations [E] and [A] are comparable (in contrast to the usual case in enzyme kinetics, where  $[E] \ll [A]$ ) then for the concentration of bound enzyme, [EA], we obtain, by straightforward solution of the binding equation and the two conservation equations  $[E] + [EA] = [E]_0$  and  $[A] + [EA] = [A]_0$ , the following expression (see also Ref. 19):

$$[EA] = \frac{[E]_0 + [A]_0 + K_a - \sqrt{([E]_0 + [A]_0 + K_a)^2 - 4[E]_0[A]_0}}{2}$$

If we assume that the enzyme activity,  $v$ , is proportional to the enzyme concentration, then the amount of bound enzyme can be assessed as:

$$v = V_0 + V_{\max}^A \cdot \frac{[E]_0 + [A]_0 + K_a - \sqrt{([E]_0 + [A]_0 + K_a)^2 - 4[E]_0[A]_0}}{2[A]_0} \quad (1)$$

where  $V_0$  is the activity of the tightly bound mitCK and  $V_{\max}^A = k_{\text{cat}} \cdot [A]_0$  is the activity obtained for  $[E]_0 \rightarrow \infty$ . This equation may be used for determination of  $[A]_0$  and  $K_a$  on the basis of the dependence of  $v$  on  $[E]_0$  (see Fig. 2 below).

If  $[A]_0 \gg [E]_0$  – and we shall henceforth limit ourselves to this case because it corresponds to the physio-

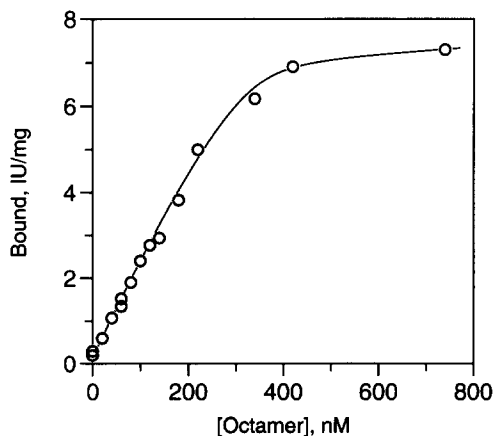


Fig. 2. Dependence of adsorbed creatine kinase activity per 1 mg of mitochondrial protein on the enzyme concentration. After water swelling and salt extraction the mitochondria were mixed with different concentrations of octameric mitCK. Medium composition: 5 mM Tris, 5 mM glycine, 1 mM  $P_i$  ( $I = 7.5$  mM), (pH 7.4), 5°C, mitochondrial protein 2.25 mg/ml. The curve is the best fit according to Eqn. 1 with parameters:  $V_0 = 0.15 \pm 0.08$  IU/mg,  $V_{\max}^A = 7.4 \pm 0.1$  IU/mg,  $[A]_0 = 310 \pm 14$  nM,  $K_a = 15 \pm 7$  nM.

logical situation for mitCK [2,9–11,20] – then Eqn. 1 simplifies to

$$v = V_0 + \frac{V_{\max}}{1 + K_a/[A]_0}, \quad V_{\max} = k_{\text{cat}} \cdot [E]_0 \quad (2)$$

(note the difference between  $V_{\max}^A$  and  $V_{\max}$ )

In general, the observed  $K_a$  will depend on the concentrations of ligands, such as hydrogen ions, and on the ionic strength because of the charges on the interacting species in the binding equilibria. The dependence of  $K_a$  on the ligand concentration ([H]) is given by:

$$K_a = K_a^{(0)} \cdot \frac{\left\{ 1 + \frac{[H]}{K_{f1}} + \frac{[H]^2}{K_{f2}K_{f2}} + \dots + \frac{[H]^n}{\prod K_{fi}} \right\}}{\left\{ 1 + \frac{[H]}{K_{b1}} + \frac{[H]^2}{K_{b1}K_{b2}} + \dots + \frac{[H]^n}{\prod K_{bi}} \right\}} \quad (3)$$

Eqn. 3 corresponds to the general scheme in Fig. 1A. If the proton binding can be described by the Hill equation, only the first and last terms in the parentheses are present:

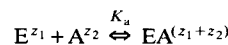
$$K_a(\text{Hill}) = K_a^{(0)} \cdot \frac{\left\{ 1 + \frac{[H]^n}{K_{f1}^n} \right\}}{\left\{ 1 + \frac{[H]^n}{K_{b1}^n} \right\}} \quad (4)$$

If, on the other hand, the binding species has  $n$  equivalent and independent sites, with site dissociation constants  $K_f$  and  $K_b$  (see Fig. 1B), then

$$K_a = K_a^{(0)} \cdot \frac{\left\{ 1 + \frac{[H]}{K_{f1}} \right\}^n}{\left\{ 1 + \frac{[H]}{K_{b1}} \right\}^n} \quad (5)$$

### The role of ionic strength

Changes in ionic strength ( $I$ ) influence the equilibrium when ions are components of the reaction, for example



In such a case the thermodynamic equilibrium (dissociation) constant  $K_0$  (at a certain pH) is

$$K_0 = \frac{[E][A]}{[EA]} \cdot \frac{10^{-(z_1^2+z_2^2)F}}{10^{-(z_1+z_2)^2F}} \quad (6)$$

where  $F$  is some form of the Debye-Hückel expression.

The concentration ratio is equal to the apparent adsorption constant  $K_a$  and we then obtain:

$$K_a = K_0 \cdot 10^{-2z_1z_2F}, \quad F = \alpha r^{1/2} / (1 + \beta \cdot r r^{1/2}) \quad (7)$$

where  $F$  is the Debye-Hückel function [21,22]:  $\alpha$  and  $\beta$  are coefficients, dependent on the temperature and the dielectric constant of the medium (at 20°C in water  $\alpha = 0.509 \text{ M}^{-1/2}$  and  $\beta = 3.29 \text{ M}^{-1/2} \text{ nm}^{-1}$ );  $r$  is the distance of closest approach between the interacting ions. Application of Eqn. 7 to macromolecules was discussed by Snyder et al. [23]. It was found that only the charges of the interacting sites, not the total charge of the species, needed to be taken into account. Analysis of the dependence of  $K_a$  on the ionic strength can therefore yield information about the charge types of the interacting sites.

#### Combined effect of a ligand and of the ionic strength

Enzyme solubilization by a substrate increasing the ionic strength may be described by the model in Fig. 1B, where H is substituted by S. Here the soluble form of the enzyme has a higher affinity for the substrate than the adsorbed form, and an increase in S causes an increase of the ionic strength. To simplify the problem we assumed the substrate to bind exclusively to the free form of the enzyme. In this case  $K_a$  from Eqn. 2 is given as:

$$K_a = K_0 \cdot \left\{ 1 + \frac{[S]}{K_S} \right\}^n \cdot 10^{-2z_1z_2F} \quad (8)$$

where  $[S]$  is the substrate concentration,  $K_S$  is the dissociation constant of S from the solubilized enzyme, and  $n$  is the number of substrate binding sites. Here the  $n$  value represents the number of substrate binding sites masked after adsorption; those that are not masked by adsorption on the membrane will not influence the binding equilibrium.

### Results and Analysis

In the analysis of the data the following protocol was adopted:

(1) Binding curves (fractional enzyme binding vs. enzyme concentration at specified pH and ionic strength) were analyzed using Eqn. 1. This yielded values of  $[A]_0$ , i.e. the concentration of binding sites on the membrane, and a value of the apparent dissociation constant  $K_a$  at the stipulated conditions.

(2) The dependence of binding on pH was analyzed, first using the 'Hill-form' Eqn. 4 to determine whether  $n$  was different from 1, and since that was found to be the case, subsequently by the use of Eqn. 5 to determine the parameters.

(3) The influence of the ionic strength on the binding of mitCK was analyzed using Eqn. 7. This yielded the product  $z_1z_2$  of the charges of the interacting sites.

(4) Analysis of mitCK solubilization by ADP and ATP requires a combination of the equations for ligand equilibria and the ionic strength effect. The applied form is Eqn. 8, which allows us to estimate the number of membrane-masked mitCK nucleotide binding sites.

#### Determination of the membrane site concentration $[A]_0$

The binding experiments were performed at pH 7.4, low ionic strength (7.5 mM) and a mitochondrial protein concentration of 2.25 mg/ml, as these conditions appeared to yield effective adsorption [7–10]. According to ultracentrifugation data more than 90% of the enzyme was in the octameric form (not shown). The creatine kinase activity stemming from the re-adsorbed enzyme was used as a measure of the adsorption. Prior to the activity determination the membrane-bound mitCK was released by  $P_i$ -buffer, see Materials and Methods. The saturation curve was analyzed by Eqn. 1 (see Fig. 2), and gave the following results (value  $\pm$  S.D.):  $V_0 = 0.15 \pm 0.08 \text{ IU/mg}$ ,  $V_{\max}^A = 7.4 \pm 0.1 \text{ IU/mg}$ ,  $[A]_0 = 310 \pm 14 \text{ nM}$ ,  $K_a = 15 \pm 7 \text{ nM}$ . The value  $[A]_0 = 310 \text{ nM}$  means that 0.14 nmol of octamer may be adsorbed on membranes containing 1 mg of mitochondrial protein.

We analyzed in the same way data from the literature on mitCK binding to the membrane [9,10,20]. We assumed that under the described conditions mitCK was an octamer with  $M_r = 340$ .  $M_r$  values in the range 150–250 are presently considered artificial [2]. The results of our analysis are presented in Table I.

#### The pH dependence of mitCK adsorption

The pH dependence was investigated under the same conditions.

The mitochondrial protein concentration was increased up to 22.5 mg/ml ( $[A]_0 = 3100 \text{ nM}$ ) in an attempt to obtain adsorption of the deprotonated enzyme. No significant adsorption of the deprotonated mitCK was seen under these conditions.

The data from Fig. 3 were fitted first by a Hill type model (Eqn. 4). We found  $n = 1.9$ – $2.2$  (Hill's coefficient for proton binding).

We then performed a more general fit of a model with  $n$  equivalent sites for proton binding using Eqn. 2 and Eqn. 5 for  $K_a$ , implying the existence of oligomeric forms of the enzyme with different affinity to protons in the free and adsorbed states. It was found – by trial and error – that an optimal value of the number of proton binding sites  $n$  was 4. This value was then subsequently used for the curve fitting. The values obtained for the parameters in Eqn. 2 and Eqn. 5 were (value  $\pm$  S.D.):  $V_0 = 0\%$  (assigned constant),  $V_{\max} = 96 \pm 3\%$ ,  $K_a^{(0)} = 0.39 \pm 0.4 \text{ mM}$ ,  $pK_f = 8.8 \pm 0.3$ ,  $pK_b =$

TABLE I

## Membrane saturation with octameric mitCK

Data were fitted according to Eqn. 1.

Reference and conditions	Adsorption sites (nmol/mg protein)	Membrane sites occurring in isolated mitochondria (%)	$K_a$ (nM)
Hall et al. [20] <sup>a</sup> pH = 7.2, $I = 4$ mM, 10°C			
heart, mitochon.	0.10	14	4.5
liver hypotonic medium	0.14	-	7.0
Lipskaya et al. [9] <sup>a</sup> pH = 7-7.4, $I = 12$ mM, 0-5°C			
heart mitochon. 0.1 M sucrose	0.33	11	36
Brooks et al. [10] heart mitoplast 25-30°C			
pH = 7.1, $I = 15$ mM	0.17	20	50
pH = 7.4, $I = 16$ mM isotonic medium			100
This work heart mitochon. pH = 7.4, $I = 7.5$ mM 5°C hypotonic medium	0.14	17	15

<sup>a</sup> The original data were given in activity units. The corresponding enzyme concentrations were calculated assuming a specific activity of 150 IU/mg.

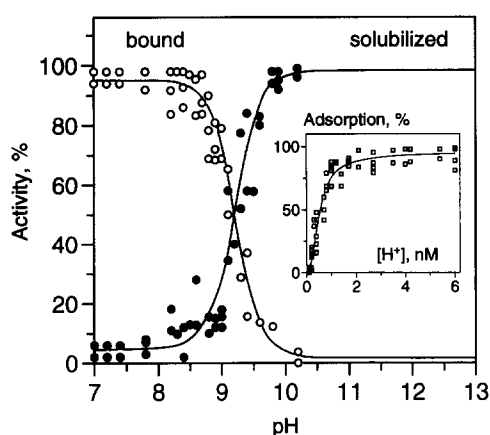


Fig. 3. pH dependence of mitCK adsorption. Medium composition as in Fig. 2 (pH 7.4),  $I = 7.5$  mM, 5°C, 22.5 mg/ml of mitochondrial protein (3100 nM of adsorption sites), 0.1 mg/ml of mitCK (290 nM of octamer). 100% corresponds to the total enzyme activity added. Activity of the tightly bound mitCK has been subtracted. Circles, mitCK activity in mitochondrial pellet after centrifugation; rectangles, mitCK activity in supernatant. (Inset) The combined data plotted together as adsorption vs.  $[H^+]$ . The best fit using Eqns 2 and 5 yielded the parameters:  $V_0 = 0\%$  (assigned),  $V_{max} = 96 \pm 3\%$ ,  $K_a^{(0)} = 0.39 \pm 0.4$  nM,  $K_f = 1.6 \pm 0.8$  nM, ( $pK_f = 8.8 \pm 0.3$ ),  $K_b = 0.16 \pm 0.08$  nM ( $pK_b = 9.8 \pm 0.3$ ).

$9.8 \pm 0.3$ . The other constants (see Fig. 1B) were calculated using the relations resulting from the principle of detailed balance:  $K_a^{(4)} = K_a^{(0)} \cdot (K_b/K_f)^4$ ,  $K_f/K_b = K_a^{(0)}/K_a^{(1)} = K_a^{(2)}/K_a^{(3)} = K_a^{(4)}$ . Hence:  $K_a^{(1)} = 39$   $\mu$ M,  $K_a^{(2)} = 3.9$   $\mu$ M,  $K_a^{(3)} = 0.39$   $\mu$ M,  $K_a^{(4)} = 39$  nM.

The same approach was applied to the data already published. Schlegel et al. [15] described octameric mitCK data for adsorption vs. pH similar to our results except for a  $K_a^{(0)}/[A]_0$  ratio of  $1.1 \cdot 10^3$ . Adsorption of the dimeric form from the same paper could be fitted by the parameters:  $K_a^{(0)}/[A]_0 = 1.8 \cdot 10^4$ ;  $K_a^{(n)} \rightarrow 0$ ,  $pK_f \rightarrow -\infty$ ,  $pK_b = 9.9$ ,  $n = 2$ . Unfortunately, an insufficient number of points in both curves precluded a reliable analysis.

The data obtained by Brooks et al. [10] were completely different, and the fitting resulted in the following parameters:  $K_a^{(0)} = 3$   $\mu$ M,  $K_a^{(n)} = 3$  nM,  $pK_f = 6.0$ ,  $pK_b = 9.0$ ,  $n = 1$  (the constants were recalculated using  $M_r = 340$ ). The value of  $n = 1$  is surprising: it implies that the octameric mitCK has only one site for  $H^+$ -binding.

The pH value of 6.8 corresponding to 50% adsorption obtained by Vial et al. [7] probably reflected ionization of the 20 mM  $P_i$ -buffer used in the experiment. This process would yield fluctuations of the ionic strength in the range 20-60 mM which is crucial for mitCK adsorption (see below).

The lack of solubilization at pH 9 observed by Lipskaya et al. [9] may be ascribed to the relatively small ratio  $K_a^{(0)}/[A]_0 \approx 10$ , causing a shift of the pH curve to a more alkaline region.

## Influence of ionic strength

The solubilization of mitCK by increasing ionic strength was attained by addition of KCl under the conditions described in the legends to Figs. 3 and 4.

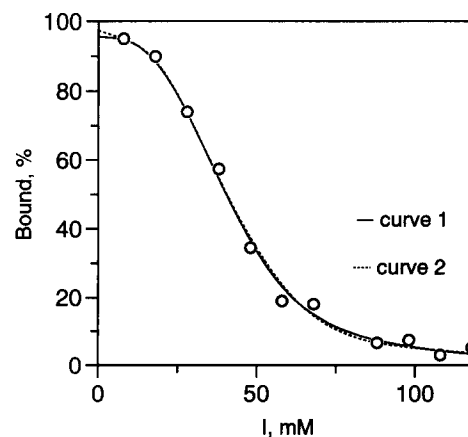


Fig. 4. Rebinding of mitCK to mitochondrial membranes in the presence of different amounts of KCl. Except for the added KCl the conditions are as described in Fig. 3. The best fit (curve 1) according to Eqn. 2 and Eqn. 7:  $V_0 = 2.9 \pm 1.3\%$  (bound activity at  $I \rightarrow \infty$ ),  $V_{max} = 93 \pm 2\%$  ( $I$ -dependent activity),  $K_0 = 0.34 \pm 0.1$  nM,  $\beta \cdot r = 1.4 \pm 0.7$   $M^{-1/2}$ ,  $-z_1 z_2 = 25.8 \pm 0.9$ . Curve 2: the fit with  $\beta \cdot r = 0$ .

TABLE II

## MitCK solubilization by the ionic strength

The parameters listed were obtained by data fitting, using Eqns. 2 and 6. To calculate  $K_0$  the amount of adsorption sites was taken as 0.15 nmol per mg mitochondrial protein.

Source, conditions at zero concentration of ligand	Solubilizing ligand	$\beta \cdot r$	$K_0$ (nM)	$-z_1 z_2$
Vial et al. [7,8] mitoplast, isotonic medium pH = 7.4, $t = 10^\circ\text{C}$ , $I = 9$ mM	KCl, MgCl <sub>2</sub> , P <sub>i</sub>	-1.1	3.15	13
		0 (fixed)	1.23	18
Lipskaya et al. [9] mitochon., 0.1 M sucrose pH = 7-7.5, $t = 0-5^\circ\text{C}$ , $I = 0$	NaCl, MgCl <sub>2</sub> , Tris, P <sub>i</sub> , CrP	1.6	0.10	24
		0 (fixed)	0.92	13
Brooks et al. [10] mitoplast, isotonic medium pH = 7.4, $t = 25-30^\circ\text{C}$ , $I = 6$ mM	NaCl	-2.5	0.69	7.4
		0 (fixed)	0.003	26
	NaCl, H <sub>2</sub> O-mitochon.	1.3	$1 \cdot 10^{-5}$	43
		0 (fixed)	$3 \cdot 10^{-7}$	26
	CrP	1.3	$9 \cdot 10^{-5}$	47
		0 (fixed)	$4 \cdot 10^{-5}$	29
This paper: mitochon., hypotonic medium pH = 7.4, $t = 5^\circ\text{C}$ , $I = 7.5$ mM	KCl	1.4	0.34	26
		0 (fixed)	2.9	15
Mean, (excl. Brooks et al.)		0 (fixed)	1.7	15.4

The data from Fig. 4 were analyzed using Eqn. 2, with  $K_a$  depending on the ionic strength as in Eqn. 7. The Debye-Hückel coefficient  $\alpha$  was accepted as  $0.49 \text{ M}^{-\frac{1}{2}}$  ( $5^\circ\text{C}$ );  $\beta \cdot r$  was used as variable parameter, to be determined by non-linear regression analysis.

The analysis yielded the following values of the parameters:  $V_0 = 2.9 \pm 1.3\%$ ,  $V_{\max} = 93 \pm 2\%$ ,  $K_0 = 0.34 \pm 0.10 \text{ nM}$ ,  $\beta \cdot r = 1.4 \pm 0.7 \text{ M}^{-\frac{1}{2}}$ ,  $-z_1 z_2 = 25.8 \pm 0.9$ , see curve 1 in Fig. 4. The data from the literature were fitted in the same manner and the results are presented in Table II.

It is seen from this table that the parameter  $\beta \cdot r$  has a rather low, and sometimes negative, value. In fact, the mean value over all the data is close to zero. A value of  $\beta \cdot r$  of the order of 1 (and a fortiori a negative value) is physically unrealistic: it corresponds to a radius of the charged particle of about 0.5 nm,

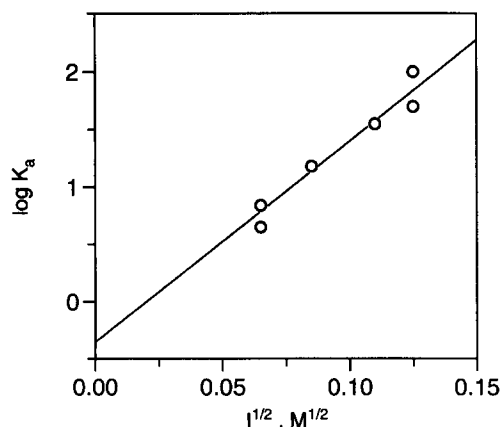


Fig. 5. Dependence of the mitCK-membrane dissociation constant on the ionic strength (conditions as in Table II). The line is the best fit according to Eqn. 9,  $K_0 = 0.43 \pm 0.19 \text{ nM}$ ,  $-z_1 z_2 = 17.5 \pm 2.0$ .

whereas the expected value for octameric mitCK would be about 10-times higher. We therefore repeated the analysis with a fixed value of  $\beta \cdot r$  of zero. This is equivalent to using the Debye-Hückel limiting law for the activity coefficients. The fits in this case are satisfactory, and statistically indistinguishable from the earlier fits (see curve 2 in Fig. 4). There are thus no statistical grounds for using the more complicated Debye-Hückel expression. The values of the parameters are included in Table II as the second entry in each table row. Excluding the values obtained from the data of Brooks et al. [10], which are anomalous in several ways, the mean value of the charge parameter is  $-z_1 z_2 \approx 16$ .

To corroborate this result we have used the adsorption constants from Table I, which were obtained in a different way. Since these were all determined at ionic strengths less than 0.02 M, we would expect the activity coefficients in these cases to be governed by the limiting law  $F(I) = \alpha\sqrt{I}$  (assuming that this theory is applicable to large protein particles), and hence a plot of  $\log K$  vs.  $\sqrt{I}$  should yield a straight line with slope =  $z_1 z_2$  and intercept =  $\log K_0$ . This is shown in Fig. 5. The regression coefficients were:  $K_0 = 0.43 \pm 0.19 \text{ nM}$ ,  $-z_1 z_2 = 17.5 \pm 2.0$ . It should be noted that the two last points (from Brooks et al.) were obtained at a temperature of  $25-30^\circ\text{C}$ . Elimination of these points from the set did not significantly change the values of parameters\*.

\* If it is true that the dissociation constants are almost independent of temperature, then this implies that the binding process is almost entirely entropy-driven.

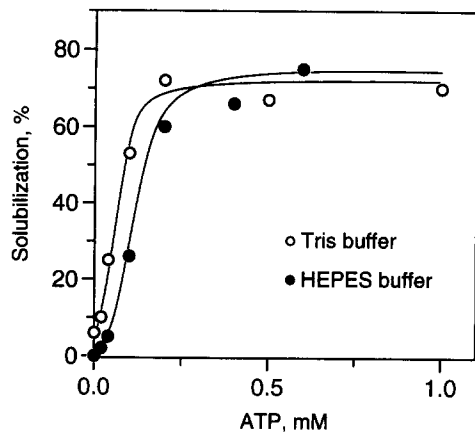


Fig. 6. MitCK solubilization by ATP (data from Vial et al. [8]). Experimental conditions: pH = 7.4,  $I = 3$  mM (in HEPES buffer) and  $I = 9$  mM (in Tris buffer + sucrose) at  $[ATP] = 0$ ,  $10^\circ\text{C}$ . Curve 1: solubilization in HEPES buffer,  $V_0 = 75 \pm 3\%$ ,  $V_{\text{max}} = -77 \pm 4\%$ ,  $K_s = 0.085 \pm 0.035$  mM. Curve 2: solubilization in Tris buffer,  $V_0 = 72 \pm 3\%$ ,  $V_{\text{max}} = -74 \pm 3\%$ ,  $K_s = 0.076 \pm 0.029$  mM. Fit using Eqn. 2 and Eqn. 8. For more details, see text.

#### Solubilization of mitCK by nucleotide substrates

We analyzed the original data [8–10] implying that subunits involved in the interaction with the membrane cannot bind substrates. The number of such subunits is assumed to be four, according to our calculations and to the known structural features of mitCK [24–26]. A typical solubilization curve is presented in Fig. 6. It was fitted using Eqn. 2 and Eqn. 8 where the ionic influence of the substrate is assumed to be governed by the limiting Debye-Hückel law with constants:  $\alpha = 0.49$   $\text{M}^{-\frac{1}{2}}$ ,  $-z_1z_2 = 16$ . The mitCK dissociation constant from membranes at  $I = 0$  was taken as  $K_0 = 1$  nM. The nucleotide site dissociation constants obtained are presented in Table III.

TABLE III

#### MitCK solubilization by nucleotide substrates

The data were fitted according to Eqns. 2 and 8.

Source, conditions at zero ligand concentration	Ligand	$K_s$ (mM)
Lipskaya et al. [9]	MgATP	1.9
pH = 7.9 or 6.8, $I = 0$ , $0-5^\circ\text{C}$	H <sub>2</sub> ATP	7.7
0.1 M sucrose		
Vial et al. [8]		
pH = 7.4, $I = 3-9$ mM, $10^\circ\text{C}$		
HEPES	ATP	0.086
HEPES + sucrose	ATP	0.028
Tris + sucrose	ATP	0.076
Tris + sucrose	ADP	0.048
Brooks et al. [10]		
pH = 7.4, $I = 6$ mM, $25-30^\circ\text{C}$	MgATP	22.0
Mops + mannitol isotonic media	MgADP	6.0

From Table III we note that, as with the pH- and  $I$ -dependence (see above), the data from Brooks et al. [10] are very different from those from other sources.

#### Discussion

There are three factors affecting mitCK binding which have been investigated independently here: the mitCK concentration, pH and the ionic strength of the medium. (The effect of temperature was not investigated.)

Our calculations showed that in some cases the standard deviation of the parameters were quite large. This is particularly true for the dissociation constants  $K_a$ ,  $K_a^{(0)}$  and  $K_0$ , where the relative S.D. values are in the range 50–100% of the mean value. Since the standard deviation of an estimated parameter in a model results from a distribution of the errors, or spread of the data, this relatively large error implies that the model is not very sensitive to the precise value of the parameter in question, which are then only estimated to within a factor of 2. The same is true for the values of  $K_f$  and  $K_b$ , the dissociation constants for H<sup>+</sup>-ion: a deviation in pK of 0.3 is equal to a deviation of a factor of two in  $K$ . Such results are not unusual in multisite binding models.

The question of the choice of expression for the activity coefficients was discussed in the previous section. We concluded that there are no statistical grounds for including a non-zero value of  $\beta \cdot r$  in the conventional Debye-Hückel expression. Also, the values obtained when included were unrealistically low and in some cases negative. It is seen, however, from Table II that even the presence of a low value of  $\beta \cdot r$  has a pronounced effect on the determination of the other constants  $K_0$  and  $z_1z_2$ : positive  $\beta \cdot r$  leads to a considerable increase in both parameters, to compensate for the increase in the denominator of the Debye-Hückel expression. Since the unrealistic values obtained for  $\beta \cdot r$  in all cases leads us to consider it merely an additional fitting parameter, with no obvious physical interpretation, we reject the more complex model on statistical grounds. We add here that a physically realistic value  $\beta \cdot r = 17$  leads to  $K_0 = 1.3 \cdot 10^{-11}$  nM and  $z_1z_2 = 313$ , which in turn are quite unreasonable. These considerations, together with the fact that the directly measured adsorption constants at low ionic strength behave according to the Debye-Hückel limiting law, lead us to conclude that the value for the parameters obtained when this function is used for all the data yields the best values for all parameters. Accordingly, we suggest that the charge parameter  $z_1z_2$  should be taken as  $-16$ .

The most natural interpretation of this latter result, when taking into account the known structural features

of mitCK as consisting of a regular octamer, is to suppose that each of the interacting sites bears a charge of 4, the positive charge being on the mitCK, and the negative charge on the binding membrane (i.e., two molecules of cardiolipin). This interpretation also is in accordance with the analysis of mitCK solubilization by ATP and ADP. These data could be satisfactorily described with a model leading to four sites of the mitCK being masked upon adsorption. This in turn implies that ATP (ADP) stabilizes mitCK in solution. Such a masking effect of the binding could not be observed, for example, by comparison of mitCK activities in a mitochondrial suspension in  $H_2O$  (bound mitCK) with those in, say 0.2 M KCl (solubilized mitCK), because MgATP (MgADP) in the reaction medium will cause fast release of originally bound enzyme in the water suspension.

Application of these results allows us to suggest a scheme for binding of mitCK to the inner mitochondrial membrane. From electron microscopy it is known that the molecule of the octameric mitCK has a cube like structure with 2 layers, each consisting of 4 subunits [2,24,25]. The 'top' and 'bottom' surfaces of the octamer capable of adsorption [2,25] have different properties, probably due to asymmetric association of the subunits [25,26]. The individual adsorption site has four equal amino acid residues essential for binding. In the free form of the enzyme these residues have  $pK_f \approx 8.8$ . In the adsorbed state the affinity to protons increases ( $pK_b \approx 9.8$ ). Therefore, the decrease in pH shifts the equilibrium towards the adsorbed state of the enzyme. The deprotonated octameric mitCK has a very low affinity for membranes ( $K^{(0)} \approx 0.39$  mM), but after protonation the affinity of the enzyme for membranes increases many times, and  $K_a^{(4)}$  becomes equal to 39 nM at  $I = 7.5$  mM.

Lysine is a likely candidate to form the adsorption site of mitCK since the  $pK$  of its side chain is 10.5. The particular roles of Arg-19 and Lys-20 have been discussed by Cheneval and Carafoli [12]. They concluded that both of the residues are inside the binding site. Our data support this conclusion, but the role of Arg-19 seems to be indirect because the guanidine group of arginine ( $pK \sim 12$ ) should be still protonated at the pH at which there is no adsorption. The general scheme of mitCK adsorption is presented in Fig. 7.

With the parameters obtained here we can predict mitCK behavior in physiological conditions. It is generally accepted that about 20% of mitCK is in a tightly bound state and thus 80% of the enzyme may be distributed between an adsorbed and a solubilized state. Assuming 0.4  $\mu$ l of intermembrane space per 1 mg of mitochondrial protein, the mitCK concentration will be 20 mg/ml. It implies the existence of only the octameric form [2,27]. One can see that less than 10% of the mitCK (in addition to the tightly bound enzyme) is

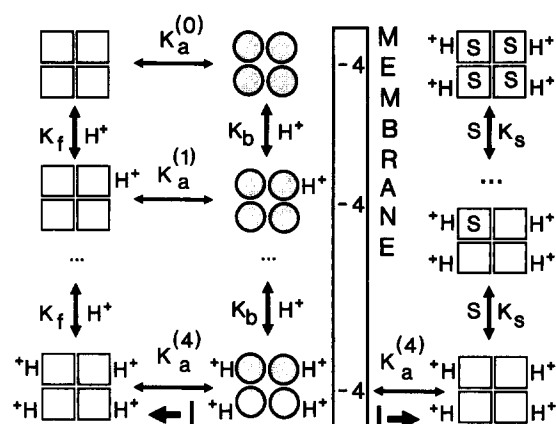


Fig. 7. MitCK binding to the mitochondrial membrane. Only one layer of a cube-like octamer with 4 subunits is presented. Another layer is hidden behind. Two opposite surfaces ('top' and 'bottom') of the cube-like molecule can interact with the membrane in the same way. The adsorption site of mitCK consists of four lysine (?) residues with  $pK_f = 8.8$  in the free enzyme and  $pK_b = 9.8$  in the bound form. The deprotonated enzyme has a very low affinity for the membranes ( $K_a^{(0)} = 0.39$  mM). Protonation of the protein leads to a remarkable increase in affinity ( $K_a^{(4)} = 39$  nM). The constants mentioned above are valid for the conditions:  $I = 7.5$  mM,  $t = 5^\circ\text{C}$ . Interacting species (mitCK and membrane) behave as if their charges were +4 and -4 respectively, according to the Debye-Hückel limiting law. The addition of nucleotide substrates (S) shifts the equilibrium towards the solubilized state of mitCK, since in the adsorbed protein the ligands have access to four specific sites and in the free protein to eight sites.

in an adsorbed state at pH 7.4,  $I = 0.2$  M,  $[\text{ATP}] + [\text{ADP}] = 5$  mM,  $-z_1z_2 = 16$ ,  $K_0/[A]_0 = 1 \cdot 10^{-6}$ .

It should be noted that the amount of tightly bound mitCK calculated according to its specific activity in isolated mitochondria may be underestimated because extramitochondrial substrates can have a limited access to mitCK inside the tight complex. Therefore the role of tightly bound mitCK is expected to be more pronounced under physiological conditions, when the enzyme is exposed to ATP from the mitochondrial matrix.

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