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Kinetics of Chemical Modification of Arginine Residues in Mitochondrial Creatine Kinase from Bovine Heart: Evidence for Negative Cooperativity

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Abstract—The kinetics of chemical modification of arginine residues in mitochondrial creatine kinase (mit-CK) from beef heart by 4-hydroxy-3-nitrophenylglyoxal (HNPG) have been studied with simultaneous registration of enzyme inactivation. Experiments showed that complete inactivation of mit-CK corresponded to modification of two arginine residues per mit-CK monomer. The data on the modification kinetics can be described by the sum of two exponential terms and suggest strong negative cooperativity in the binding of HNPG to arginine residues. The rate constants for the fast and slow phases of modification differ by a factor of about 50. The corresponding rate constants for inactivation differ by a factor of about 30. The rate constant for the slow stage of inactivation is twice as large as that for the rate constant for the slow stage of modification, i.e., the inactivation process is ahead of the modification process.

Key words: mitochondrial creatine kinase, inactivation, modification, essential arginine residues, negative cooperativity

Creatine kinase (CK) catalyzes the reversible transfer of a phosphoryl group from ATP to creatine with the formation of ADP and creatine phosphate, the latter being a depot of macroergic phosphates in the cell [1-5]. The enzyme has been found in the cell in the form of three cytoplasmic isoenzymes (cyt-CK), which are binary combinations of identical subunits (MM, BB) or different subunits (MB) [2, 4], and two mitochondrial isoenzymes (mit-CK), which exist as dimers and octamers [6, 7].

X-Ray analysis of chicken mit-CK [8] and latter analysis of cyt-CK of the muscle type (MM) [9] and brain type (BB) [10] have shown that the subunit of CK consists of small (1-112) and large (113-380) domains. In the cleft between the two domains, an active site is located. The amino acid residues of the active site involved in catalysis and the binding of the substrates have been identified using a number of physicochemical methods including the method of chemical modification. Among these are cysteine, arginine, lysine, histidine, tryptophan, and aspartic acid [4-6]. In recent years, additional information on the important role of Cys282 [11-13], Arg291 [12], Trp227 [14], Trp210 [14, 15], His295 [16], and His65 [17] in the binding of the substrates and structural organization of CK has been obtained using the method of site-directed mutagenesis. It is of interest that Cys282, Arg291, His295, and Trp227 are the same residues whose role has been established by the method of chemical modification.

The essential role of arginine residues in the functioning of cyt-CK was first described by Borders and Riordan [18]. These investigators showed that modification of one arginine residue per subunit of cyt-CK from rabbit muscles resulted in 100% loss in the catalytic activity and capability for the binding of the nucleotide substrates [18, 19].

Previously we demonstrated 100% inhibition of mit-CK from beef heart as a result of modification by 2,3-butanedione and 4-hydroxy-3-nitrophenylglyoxal (HNPG) [20, 21]. We showed that the complete inactivation of the enzyme corresponded to modification of two arginine residues per monomer, but not modification of one arginine residue as in the case of cyt-CK [21]. The substrates MgATP and MgADP protected the enzyme from inactivation [20].

Abbreviations: HNPG) 4-hydroxy-3-nitrophenylglyoxal; mit-CK) mitochondrial creatine kinase; cyt-CK) cytoplasmic creatine kinase.

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On the basis of the data on chemical modification of cyt-CK from rabbit muscles by phenylglyoxal in combination with mass spectrometry, Wood et al. [22] came to the conclusion that phenylglyoxal interacted with three arginine residues (Arg291, Arg129, and Arg131). Lastly, X-ray study of chicken mit-CK with resolution of 3 Å showed the presence of five arginine residues (at positions 287, 125, 127, 315, and 336) in the region of the binding of the three-phosphate-containing fragment of the ATP molecule [8].

Thus, at the present time the question of what are the number and functional role of arginine residues localized in the active site of CK is still unclear.

In the present work, we first studied the kinetics of modification of arginine residues in mit-CK from beef heart by HNPG and the kinetics of inactivation of the enzyme. It was shown that modification of two arginine residues per monomer is necessary for the complete inactivation of mit-CK. The analysis of the kinetics of modification of two arginine residues by HNPG revealed well-marked negative cooperativity: their modification rate constants differed by a factor of about 50. It was shown that inactivation proceeds more rapidly than the modification process.

MATERIALS AND METHODS

Creatine kinase was isolated from beef heart mitochondria according to the procedure described in [23, 24]. The protein concentration was determined by the method by Bradford with Coomassie blue G-250 [25] and spectrophotometrically at 260 and 280 nm.

Activity of mitochondrial creatine kinase was determined at 30°C in the direction of the formation of ATP and creatine by registration of the change in pH of the incubation medium using a pH meter connected with a potentiometer. The reaction mixture (4 ml) contained 10 mM sodium borate buffer (or 10 mM Tris-HCl buffer), pH 7.4, 10 mM KCl, 3.3 mM $Mg(CH_3COO)_2$, 6 mM creatine phosphate, 0.5 mM ADP, and 0.2 mM dithiothreitol.

4-Hydroxy-3-nitrophenylglyoxal was synthesized according to the method by Borders and coauthors [19, 21]. The melting point for the preparation obtained was 96°C. The absorption spectrum was characterized by maxima at 316 and 395 nm (pH 8.7). The degree of purification was 95%.

Modification of mitochondrial creatine kinase (0.3 mg/ml) was carried out in 50 mM sodium borate buffer, pH 8.7, containing 0.2 mM dithiothreitol by the addition of a freshly prepared solution of HNPG (16-24 mM) in the above buffer. The final concentrations were the following: 6 μM mit-CK monomer and 2 or 4 mM HNPG. The mixture of the enzyme with modifier was incubated at 20°C for a definite time. To separate the modified protein from the excess of the inhibitor, aliquots (0.5 ml) were withdrawn and placed on the column (1.3 × 10 cm) with Sephadex G-25 equilibrated with 50 mM sodium borate buffer, pH 8.7, containing 0.2 mM dithiothreitol. Elution was carried out in the above buffer. The protein concentration and creatine kinase activity were determined in eluates. In addition, the absorption spectra were registered in the region from 240-450 nm using spectrophotometers Beckman DU-8 (USA) and Hitachi-557 (Japan). The concentration of HNPG bound to the protein was calculated from absorbance at 316 nm using the extinction coefficient of $1.09 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$. When performing calculations, data on equimolar stoichiometry of interaction of phenylglyoxal with guanidinium group of arginine of CK [22] were used. We took into account also the absorption of the protein at various protein concentrations for the given wavelength (the molecular mass of subunit of CK was taken to be 43 kD).

The rate constants for inactivation and modification were determined using the method of analysis of kinetic data proposed by Ray and Koshland [26]. The kinetic

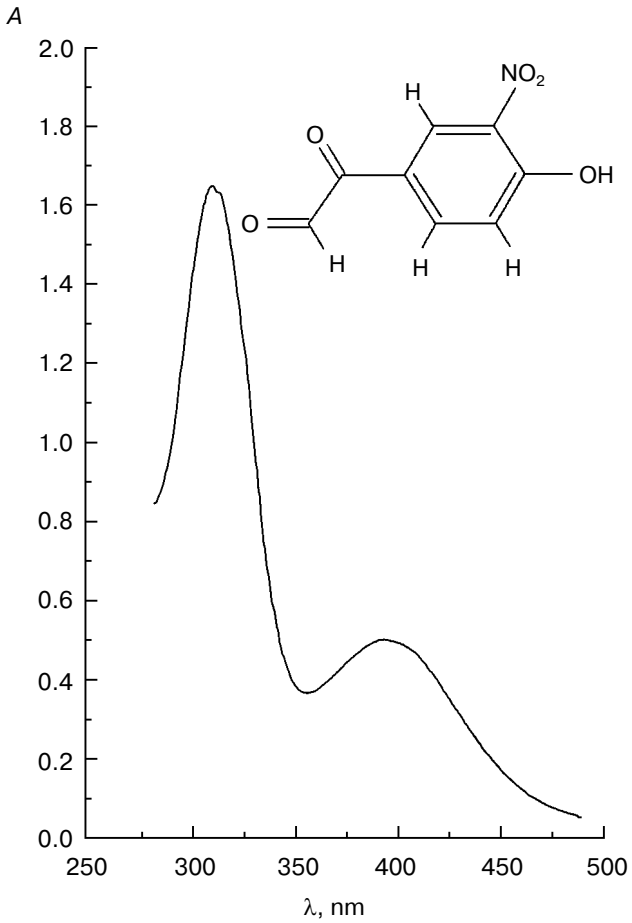

Fig. 1. Absorption spectrum of 4-hydroxy-3-nitrophenylglyoxal in 50 mM sodium borate buffer, pH 8.7.

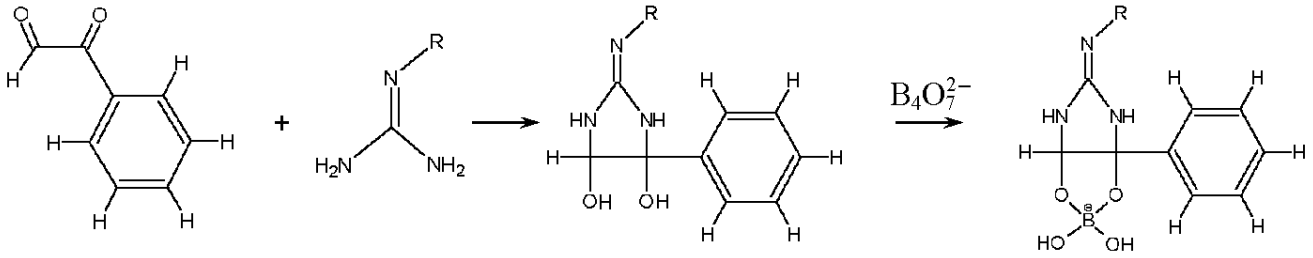

Fig. 2. Tentative mechanism of interaction of phenylglyoxal with guanidinium group of arginine residues [22] and stabilization of the compound formed by $B_4O_7^{2-}$.

curve of inactivation of mit-CK accompanying modification by HNPG was described by the sum of two exponential terms:

$$A_t/A_0 = \alpha_1\exp(-k_1t) + \alpha_2\exp(-k_2t), \quad (1)$$

where A_0 and A_t are the initial and current values of the activity; k_1 and k_2 are the rate constants of pseudo-first order for the "fast" and "slow" phases; α_1 and α_2 are the portions of the activity corresponding to the "fast" and "slow" phases, respectively.

The time course of modification of the functionally important amino acid residues has the following form:

$$1 - P_t/P_\infty = \beta_1\exp(-k_1t) + \beta_2\exp(-k_2t), \quad (2)$$

where P_∞ and P_t are the maximum and current number of arginine residues modified by HNPG; k_1 and k_2 are the rate constants of pseudo-first order for the "fast" and "slow" phases; β_1 and β_2 are the portions of the modified residues for the "fast" and "slow" phases, respectively.

The mathematical treatment of the kinetics of inactivation and modification was carried out using the program Origin 5.0 (Microcal Software Inc., USA).

To study modification of arginine residues in mit-CK and simultaneously proceeding inactivation of the enzyme, a chromophore-containing derivative of phenylglyoxal, namely, HNPG, was used. The fact that HNPG possesses a characteristic spectrum in the visible region (Fig. 1), allows the amount of HNPG bound to mit-CK to be spectrophotometrically determined. Phenylglyoxal modifies the guanidinium group of arginine residues with the formation of a compound that is stable in the presence of borate-anion $B_4O_7^{2-}$ (Fig. 2). Therefore, in the present work we used 50 mM sodium-borate buffer, pH 8.7, since for this buffer the equimolar stoichiometry of interaction of phenylglyoxal with arginine residues of the proteins has been demonstrated [27]. It should be noted that the equimolar stoichiometry of modification of arginine residues for cyt-CK from rabbit muscles was observed also in and Bicine buffers [22].

The following reagents were used in the present work: ADP, dithiothreitol, and Coomassie blue G-250 from Serva (Germany) and creatine phosphate from Reanal (Hungary). Other reagents were of domestic production ("chemically pure" and "highest quality" grade). Tris, borax ($Na_2B_4O_7$), and boric acid (H_3BO_3) were recrystallized.

RESULTS

The study of the kinetics of inactivation of mit-CK by HNPG without removal of the inhibitor has shown that the experimental points are satisfactorily described by the theoretical equation involving two exponential terms (Fig. 3). The second order rate constants for the "fast" and "slow" phases differ by a factor of 30, i.e., a value greater than an order of magnitude (Table 1). As can be seen from Fig. 3, the kinetic curve involves a rapid stage of inactivation with duration of about 2 min fol-

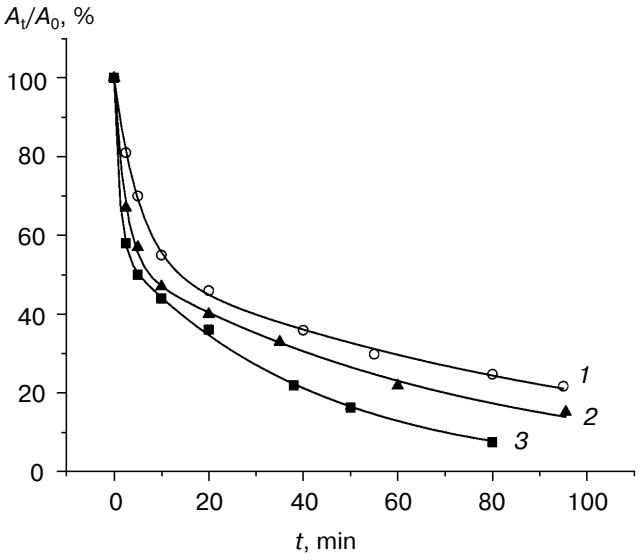

Fig. 3. Kinetics of inactivation of mit-CK (7 μM calculated per subunit) in 50 mM sodium borate buffer, pH 8.7, containing 0.2 mM dithiothreitol and various concentrations of HNPG: 1.5 (*1*), 3.0 (*2*), and 6 mM (*3*). A_0 and A_t are the initial and current values of the enzymatic activity of CK.

Table 1. Rate constants for the fast (k_{fast}) and slow (k_{slow}) phases of inactivation of mit-CK by HNPG determined without the removal of the inhibitor

No.	HNPG, mM	k, $M^{-1} \cdot min^{-1}$		α_1*, %	α_2*, %
		k_{fast}	k_{slow}		
1	1.5	121 ± 9	6.4 ± 0.4	47 ± 1.8	53 ± 1.7
2	3	139 ± 11	4.6 ± 0.3	46 ± 2	53 ± 1.6
3	6	151 ± 4	4.1 ± 0.1	43 ± 1.2	56 ± 1.0
4	average	137 ± 8	5.0 ± 0.3	45 ± 1.7	54 ± 1.5

* α_1 and α_2 are the portions of the enzymatic activity corresponding to the "fast" and "slow" phases of the inactivation process.

Table 2. Number of arginine residues of mit-CK modified by HNPG after the removal of the excess of the inhibitor in the samples with different time of incubation

Region of measurement	Experiment number	Time of incubation, min	A_t/A_0*, %	Concentration of HNPG bound to mit-CK, μM	Concentration of mit-CK monomers, μM	Number of arginine residues per monomer of mit-CK
I	1	5	56	2.1	2.6	0.81
	2	6	61	2.2	2.6	0.85
II	1	55	29	2.3	2.0	1.15
	2	50	37	2.94	2.8	1.05
III	1	210	8.5	3.94	2.8	1.41
	2	210	12	3.2	2.2	1.46

* A_0 and A_t are the initial and current values of the enzymatic activity of mit-CK. In experiments No. 1 and 2, the values of A_0 are 62 and 110 units/mg, respectively. The conditions of the experiments: CK (6 μM calculated per monomer) was incubated with 2 mM HNPG.

lowed by a slow stage. Complete inactivation occurs within two and more hours.

To study the kinetics of inactivation and modification of mit-CK by HNPG, the enzyme (6 μM calculated per subunit) was incubated with 2 mM HNPG. Table 2 shows the results of two typical experiments. As can be seen from Table 2, the enzymatic activity decreases and the number of arginine residues modified by HNPG increases with increasing time of incubation of mit-CK with the inhibitor. The decrease in the activity by 80-90% corresponds to modification of 1.5 arginine residues per monomer.

To obtain the time courses of inactivation and modification processes, we performed six experiments in each series of measurements at HNPG concentrations equal to 2 and 4 mM. The experimental points in Fig. 4a are averages of 2-3 measurements. The theoretical curves (solid lines) are calculated from 23 averaged experimental points. We used a rather great number of experimental points because the kinetic experiment involves the stage of gel filtration, which introduces an error in measuring the time of incubation of the protein–inhibitor mixture. The latter is calculated as a time of protein elution from the column. In spite of the maximum standardization of the experimental conditions (the free volume of the column was 4.4 ml; the elution rate 1.3 ml/min; the volume of elution fraction 1.1-1.2 ml), the differences in the elution time for the parallel experiments were within 2 min. Therefore, the scatter of points in such a type of experiments is higher than that in the experiments where inactivation was measured without removal of the inhibitor. Nevertheless, the kinetic data on inactivation are satisfactorily described by the theoretical equation containing two exponential terms (Fig. 4a, experimental points and solid line on curve *1*). The second order rate constants for the "fast" and "slow" phases (Table 2) agree well with those obtained without the removal of the inhibitor (Table 1); the latter in turn may be considered as a control for the experiments carried out with the removal of the excess of HNPG by gel filtration. The inactivation process is accompanied by the increase in the number of arginine residues (P_t) being modified in each monomer of mit-CK (Fig. 4a, curve *2*). Fast inactivation with amplitude of 50% (<2 min) is followed by a very slow stage, which is

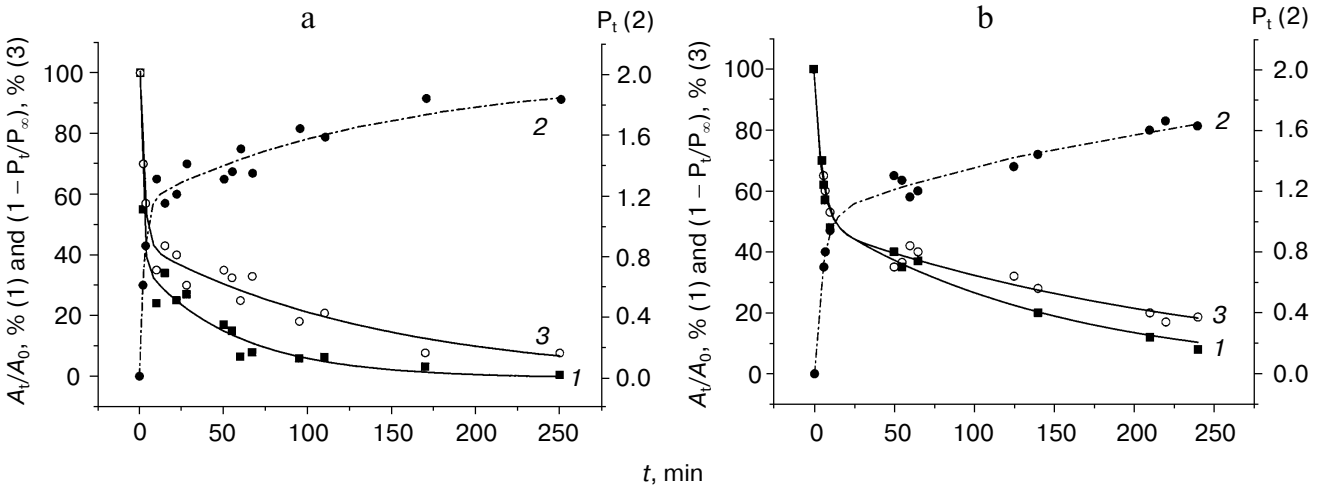

Fig. 4. Kinetics of inactivation of mit-CK by HNPG (*1*) and the corresponding amount of modified arginine residues P_t (*2*). Curve *3* shows the kinetics of the diminishing of the amount of essential arginine residues with time. Mit-CK (6 μM calculated per subunit) was incubated in 50 mM sodium borate buffer, pH 8.7, containing 0.2 mM dithiothreitol in the presence of 4 mM (a) and 2 mM (b) HNPG. The excess of the inhibitor in aliquots withdrawn at certain instants in time was removed by gel filtration.

completed in 250 min with full inactivation of the enzyme and modification of 1.9 arginine residues per monomer. This value is close to 2, i.e., the value determined earlier when establishing the stoichiometric dependence of the residual activity on the number of modified groups [21]. We used the limiting value of the modified residues (P_∞ = 2) to calculate the values of $(1 - P_t/P_\infty)$ for each time *t*. On the basis of these values, curve *3* in Fig. 4a characterizing the diminishing of the number of arginine residues essential for the enzymatic activity in the process of inactivation of mit-CK by HNPG was constructed. The rate constants for modification of mit-CK by HNPG have been calculated from the dependence of $(1 - P_t/P_\infty)$ on time.

As can be seen from Table 3, the rate constants for the "fast" and "slow" phases of the inactivation process differ by a factor of 30. The rate constants for the "fast" and "slow" phases of modification of arginine residues differ by a factor of about 50. The rate constant for the fast phase of inactivation is about 1.5 times higher than that for the fast phase of the modification process, whereas for the rate constants of the slow phases of inactivation and modification, the ratio of the rate constants is as great as 2-2.5. These differences in the rate constants indicate that inactivation of mit-CK by HNPG proceeds with higher rate than modification of arginine residues. The higher rate of inactivation is especially marked for the slow stage. As can be seen from Fig. 4a, modification of 1.5 arginine residues per monomer (or 3 residues per dimer in the octameric molecule) results in enzyme inactivation by 80-90%.

The similar results were obtained at less concentration of the inhibitor (2 mM) and the same concentration of the monomers of mit-CK, namely, 6 μM (see Fig. 4b and Table 4).

Table 3. Rate constants for the fast and slow phases of inactivation and modification of mit-CK by HNPG (4 mM) and the corresponding fractions of the phases

Parameter	Inactivation		Modification	
	k_{fast}	k_{slow}	k_{fast}	k_{slow}
Constant, $M^{-1} \cdot min^{-1}$	147 ± 29	4.6 ± 0.8	100 ± 21	1.8 ± 0.3
Fraction* of the phase, %	α_1	α_2	β_1	β_2
	62.8 ± 4.8	37.2 ± 4	56.8 ± 5.3	43.6 ± 3.2

* The fractions of the fast and slow phases of inactivation correspond to the values of α_1 and α_2 in Eq. (1). The fractions of arginine residues modified by HNPG in the fast and slow phases correspond to the values β_1 and β_2 in Eq. (2) (see "Materials and Methods").

Table 4. Rate constants for the fast and slow phases of inactivation and modification of mit-CK by HNPG (2 mM) and the corresponding fractions of the phases

Parameter	Inactivation		Modification	
	k_{fast}	k_{slow}	k_{fast}	k_{slow}
Constant, $M^{-1}\cdot min^{-1}$	119 ± 28	3.4 ± 0.5	96 ± 14	2 ± 0.2
Fraction* of the phase, %	α_1	α_2	β_1	β_2
	48.4 ± 5.6	52.2 ± 4.6	51.7 ± 3.8	48.5 ± 2.7

* The fractions of the fast and slow phases of inactivation correspond to the values of α_1 and α_2 in Eq. (1). The fractions of arginine residues modified by HNPG in the fast and slow phases correspond to the values β_1 and β_2 in Eq. (2) (see "Materials and Methods").

DISCUSSION

On the basis of the simultaneous investigation of the kinetics of modification of arginine residues and the kinetics of inactivation of mit-CK from beef heart, the following two basic results have been obtained. First, modification of two arginine residues per monomer provides full inactivation of the enzyme. Second, the kinetics of modification is characterized by a well-marked negative cooperativity in respect to binding of the inhibitor (HNPG).

The molecule of mit-CK from beef heart is an octamer, which can be considered as a tetramer consisting of weakly interacting dimers [6, 8, 23, 24]. The electron microscopy investigations of mit-CK from beef heart indicate that octameric forms are structurally labile [28, 29]. Besides, it was shown that the interactions between subunits in the dimer were extremely strong for practically all the isoenzymes [8-10]. Dissociation of dimers into monomers occurs under rigorous conditions and is accompanied by partial unfolding of the monomers.

At the present time, there are different views of the way the subunits perform their catalytic function. It is still unclear whether there are cooperative interactions between subunits [30-34] or subunits function independently of one another [35]. Previously, using mit-CK from beef heart as an example, we observed negative cooperativity with respect to the inhibitor in each dimer of the octameric form in experiments on modification of single essential cysteine residue by 5,5′-dithiobis-(2-nitrobenzoic acid) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole in the absence as well in the presence of an analog of the transition state complex (E-MgADP-NO_3^--creatine) [24, 31]. In the present work, the effect of negative cooperativity in the binding of HNPG by arginine residues has been demonstrated.

Our results show that each dimer of mit-CK contains four essential arginine residues, two per each subunit. Recently it was shown that phenylglyoxal modified only Arg291 and Arg129 (or Arg127) in cyt-CK [22]. (Arg95 participating in the binding of creatine is not modified by phenylglyoxal [36].) In mit-CK, these residues correspond to Arg287 and Arg127 (or Arg125) [8]. Previously, we showed that it was precisely these residues that were protected by MgADP and MgATP from modification by arginine-specific inhibitors [20]. Therefore, we conclude that in our experiments with mit-CK, HNPG modifies Arg287 and another arginine residue (for example, Arg127).

The following assumptions can be put forward to explain the 50-fold difference in the rate constants for modification of two arginine residues.

First, the reason for the different reactivity of arginine residues in each of the subunits of the dimer (Arg287 is modified rapidly, whereas Arg127 is modified slowly) lies in the initially different microenvironment or the change in microenvironment of Arg127 after modification of "fast" Arg287. In this case, subunits of each dimer can function independently. In our opinion, this assumption is unlikely, if one takes into account the data on modification of the single essential residue Cys278 in mit-CK [24, 31].

Second, both arginine residues (Arg287 and Arg127) in one subunit of the dimer are modified by HNPG rapidly, the rates of their modification being approximately the same. Modification of the above arginine residues results in inactivation of one subunit and marked conformational changes in the dimer. These changes interfere with the interaction of the inhibitor with Arg287 and Arg127 in the second subunit. In this case, the great difference in the rate constants for each phase of modification and inactivation is due to negative cooperativity. The data on X-ray studies of the crystals of dimeric CK from *Torpedo californica* grown in the presence of MgADP, nitrate, and creatine [30] support the second assumption. Negative cooperativity in the binding of these ligands has been demonstrated. One subunit binds all the ligands with the formation of the abortive complex E-MgADP-NO_3^--creatine, whereas the second subunit binds only MgADP.

Results obtained in the present work provide new evidence for the non-equivalence of subunits of CK on the binding of various ligands. The problem of the interaction of CK subunits continues to be intensively studied by using chemically [32, 33, 35] or genetically [34] modified subunits.

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