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MAMMALIAN 3-OXOSTEROID Δ4-Δ5-ISOMERASE

A MEMBRANE-BOUND ENZYME
II. ACTIVATION BY DIVALENT CATIONS

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Summary

Alkaline-earth ions (Mg^{2+}, Ca^{2+} and Sr^{2+}) have two specific effects on the kinetic parameters of the beef adrenal 3-oxosteroid Δ4-Δ5-isomerase activity in the microsomes and in the particles obtained after disrupting the membrane structure by action of 1 M $MgCl_2$. On the microsomal enzyme, a 2-fold increase of V is observed with the three cations under study. The small difference in the effect of the three ions could be related to their hydration energy. It is suggested that the interaction of the ion with water is the determinant step of the activation mechanism and not the fixation of the ion on the enzyme or on some others possible binding sites in this system.

With the enzyme in the proteolipidic particles, the use of EDTA as a chelating agent for the cations present in the enzymatic assay, allows the characterization of two effects: at low concentration of EDTA, an increase of K_m is observed and at higher concentration (2 mM), V is decreased. A subsequent addition of Mg^{2+} leads to an activation in two steps: V is increased in the first step without change in K_m, the second step consists of a decrease of K_m without any change in V. A relation between the structural perturbations induced by the ions (Gallay, J., Vincent, M. and Alfsen, A. (1975) Biochim. Biophys. Acta 397, 489—500) and their kinetic effect on the enzymatic reaction is established.

Introduction

The fluorescence studies on the microenvironment of different probes in microsomes and in proteolipidic particles have demonstrated a different effect of Mg^{2+} on the global lipidic phase and on the isomerase enzymatic binding site [1], which is known to be phospholipid dependent [2]. In order to corre-

late these structural changes with the catalytic properties of the studied enzyme, the effects of alkaline-earth ions (Mg^{2+}, Ca^{2+} and Sr^{2+}) on the isomerase kinetic parameters have been studied in both systems.

Materials and Methods

Chemical reagents. 5-Androstene-3,17-dione (5-androstenedione), 5-pregnene-3,21-dione (5-pregnenedione) (3—4% 4-en-3-oxosteroid) and 17β-hydroxy-19-nor-4-androsten-3-one (19-nor-testosterone) were obtained from Roussel-UCLAF and used as supplied. Divalent salts were Merck Chemical reagents, analytical grade. Ethylenediamine tetraacetic acid (EDTA) was Baker Chemicals reagent, analytical grade. All other chemicals analytical grade were purchased from commercial sources.

Enzymatic preparation. The preparation of the microsomes and of the proteolipidic particles has been previously described [1,3]. Protein determination was performed according to Lowry et al. [4] in presence of sodium dodecyl sulfate with ovalbumin as standard.

Kinetic measurements. The enzymatic assay has been described elsewhere [3]. In the microsomes, the initial velocity of the enzymatic reaction has been measured after 1 min incubation of the membranes. The assay buffer was 20 mM Tris · HCl (pH 8.5), 3.3% (v/v) dioxane. The substrates used were 5-androstenedione and 5-pregnenedione, 19-nor-testosterone has been used as an inhibitor of the microsomal activity.

In the case of the proteolipidic particles system, it has been observed that a precipitation occurs in function of time upon elimination of the Mg^{2+} [3]. Consequently the enzymatic assay must be performed with a minimum concentration of Mg^{2+} which was 3.3 mM in our experimental conditions. It is to be noticed that no aggregation can be observed during the time course of the kinetic runs (3—4 min). In some experiments, in order to decrease the Mg^{2+} concentration be low 3.3 mM, EDTA has been used as a chelating agent. In these experiments, 5-androstenedione has been used as the only substrate.

In both systems (microsomes and proteolipidic particles), K_m and V were determined according to Lineweaver and Burk [5]. Inhibition constant of 19-nor-testosterone has been obtained by Dixon plots [6].

All the kinetic measurements were carried out with a Gilford 2400 spectrophotometer at room temperature.

Results

Effect of divalent cations on microsomal activity

(1) Initial velocity studies. Microsomes have been prepared in a Tris · HCl buffer containing 5 mM $CaCl_2$. After dilution in the enzymatic assay, the concentration of $CaCl_2$ becomes 1.66 μM. In order to measure the effect of this low cation concentration on the enzymatic activity, kinetic runs have been performed in presence of various concentrations of EDTA (between 0.3 and 3 mM). No modification of the enzymatic activity has been observed in this concentration range. This indicates that the microsome-bound Ca^{2+} is not effective on the enzymatic activity.

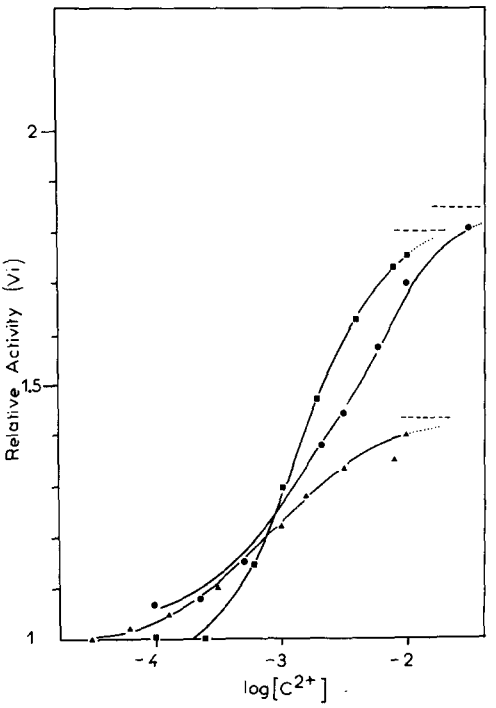

Fig. 1. The activation of microsomal isomerase by Sr^{2+} (▲), Ca^{2+} (■) and Mg^{2+} (●). Relative activity is plotted versus the logarithm of ionic concentration. The reference initial velocity, expressed as the change in absorbance per min at 248 nm, is measured without ion in the assay. The initial concentration of 5-androstenedione was 117 μM in 20 mM Tris · HCl (pH 8.5), 3.3% dioxane. The temperature was 26°C.

Experiments have been carried out in presence of increasing salt concentrations ($CaCl_2$, $MgCl_2$ and $SrCl_2$) in the enzymatic assay with either 5-androstenedione or 5-pregnenedione as substrate. The conditions are described in Materials and Methods. The basic activity has been taken as the activity of the microsomes without added ion in the assay. The results are described in Fig. 1 where the relative initial velocity for 5-androstenedione as substrate is plotted versus the logarithm of the cationic concentration. The initial velocity increases significatively with all three cations. In order to control that the reaction with the ions is reversible, EDTA has been added to the enzymatic assay in presence of 2 mM Ca^{2+}. The measured activity at 3.5 mM EDTA is the same as the initial one without added ions.

When using a reciprocal plot of the relative activity versus cationic concentration, a straight line is obtained. The intersection with the ordinate gives the value of the maximum activation at infinite concentration of the ions.

The same type of curve is obtained whatever the substrate (5-androstenedione or 5-pregnenedione). The order of efficiency of activation of the ions is $Sr^{2+} < Ca^{2+} < Mg^{2+}$. The increase of activity is 1.4, 1.8 and 1.85 times with 5-androstenedione and 1.8, 2.3 and 2.4 times with 5-pregnenedione, respectively, for each ion.

Since it has been shown that the reaction of the cations with the protein is

reversible, the cation enzyme association constant may be written according to Fridovich [7]:

$$E + nC^{2+} \rightleftharpoons EC_n^{2+}$$

(E means sensitive sites on the enzyme).

$$K_A = \frac{(EC^{2+})}{(E) \cdot (C^{2+})^n}$$

If V_0 is the activity in absence of cation, and V the activity measured at a given ionic concentration, then the increase of activity $V - V_0$ is proportional to EC_n^{2+}. Similarly if V_s is defined as the activity in presence of an infinite concentration of cation determined by the preceding reciprocal plot, the concentration of free enzymatic sites is proportional to $V_s - V$.

$$K_A = \frac{(V-V_0)}{(V_s-V) \cdot (C^{2+})^n}$$

Using the logarithmic form of the preceding equation, according to Johnson et al. [8], it follows:

$$\log K_A + n \log C^{2+} = \log \frac{V-V_0}{V_s-V}$$

If $\log[(V-V_0)/(V_s-V)]$ is plotted versus $\log C^{2+}$ a straight line is obtained, whatever the substrate. The slope gives the number of mol of activator bound per sensitive site. The intercept with the ordinate gives the value of K_A. Such a treatment of our data leads to the conclusion that only one cation is effective per sensitive site. K_A is therefore equal to the reciprocal of the cation concentration required to cause 50% activation.

The apparent dissociation constants obtained with 5-androstenedione as substrate are K_D = 0.6, 0.4, and 1.6 mM, respectively, for Sr^{2+}, Ca^{2+} and Mg^{2+}. With 5-pregnenedione, the values of K_D are: 2, 1.5 and 4 mM for the same ions.

(2) Michaelis constant and maximum velocity of the enzymatic reaction in presence of ions. Kinetic measurements have been performed without ions and in presence of 10 mM $CaCl_2$, $MgCl_2$ or $SrCl_2$. The substrate used in these experiments was 5-androstenedione. Experiments were also carried out in presence of 19-nor-testosterone, a competitive inhibitor of the isomerase activity. The results are summarized in Table I. The inhibition constant (K_I) of 19-nor-testosterone is not affected by the presence of the ions in the enzymatic assay, in agreement with the unchanged value of K_m in the same ionic conditions, but V is increased by about two times.

If 5-pregnenedione is used as substrate, the same effect is observed. In presence of ions, V is increased, e.g. V = 2400 nM · mg^{-1} · min^{-1} in presence of 10^{-2} M Ca^{2+}, which is 2.8 times the value obtained without ion in the assay. K_m remains unchanged with a value of 23 μM.

Effect of Mg^{2+} on the enzymatic activity of the proteolipidic particles

(1) EDTA inhibition. In order to understand the role of divalent cations on the structure and activity of the solubilized enzyme, experiments have been

TABLE I

THE ACTIVATION OF THE MICROSOMAL ISOMERASE BY DIVALENT CATIONS

Initial velocities were determined with 5-androstenedione as substrate. K_m and V have been determined according to Lineweaver and Burk [5], K_i of 19-nor-testosterone has been obtained from Dixon [6] representation, the apparent dissociation constant of the ions (K_D app) and the number of ions bound at sensitive sites on the enzyme, were calculated from Johnson et al. [8].

Ions (10^{-2} M)	No ion added	Sr^{2+}	Mg^{2+}	Ca^{2+}
K_m (μM)	130	130	130	120
V (nM · min^{-1} · mg^{-1})	445	745	870	925
K_i (μM)	11	10	10	11
K_D app (mM)	—	0.6	1.6	0.4
n	—	1	1	1.3

carried out with increasing concentrations of EDTA in the assay. As shown in Fig. 2a, the initial velocity change of the enzymatic reaction in function of EDTA concentration is biphasic, with a discontinuity at 1.8 mM EDTA. At this concentration the inhibition is of about 40%. The maximum inhibition is obtained for EDTA concentrations above 4 mM. The residual activity is about 30%.

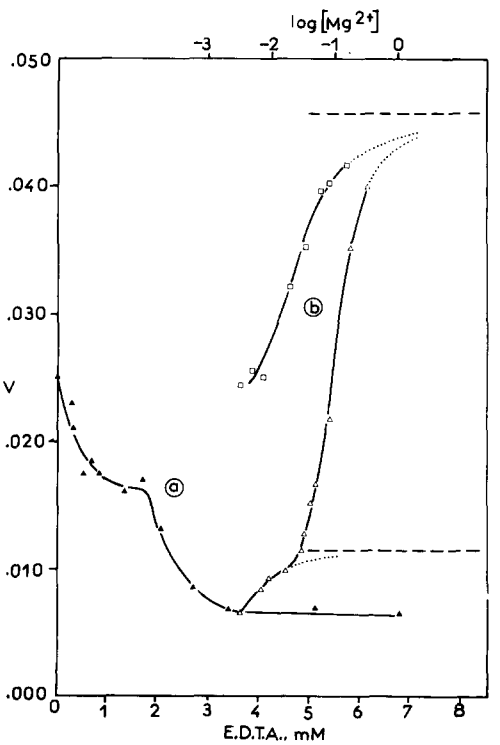

Fig. 2. (a) The inhibition of the isomerase activity of the proteolipidic particles by EDTA. Initial velocity is plotted versus EDTA concentration (▲). (b) The influence of Mg^{2+} on the activity of the isomerase in the proteolipidic particles. Initial velocity is plotted versus the logarithm of Mg^{2+} concentration. In these experiments, the EDTA concentration was either 3.3 mM (△), or no EDTA was added (□). Conditions of assay as described in Materials and Methods.

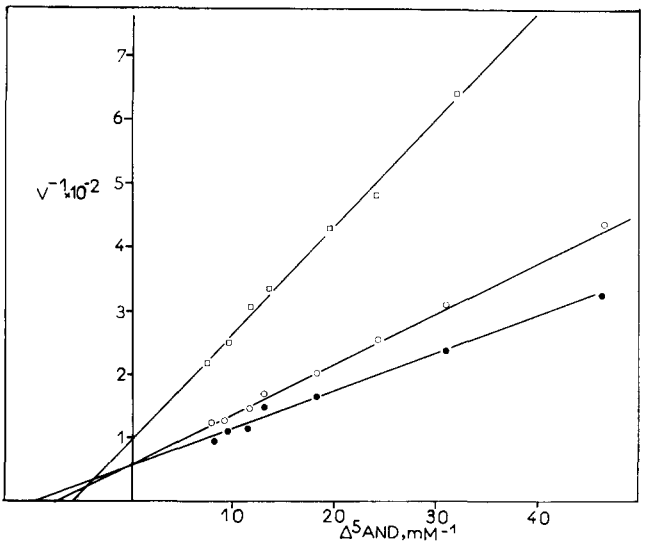

Fig. 3. The inhibition of the isomerase activity in the proteolipidic particles by EDTA. Reciprocal initial velocity is plotted versus reciprocal 5-androstenedione concentration. In these experiments, the concentration of EDTA were either 2 mM (○), 3.3 mM (□) or no EDTA was added (●). Conditions of assay as described in Materials and Methods.

Analysis of the kinetic parameters indicates that the initial part of the curve is correlated with an increase of K_m without any change in V. The part of the curve corresponding to EDTA concentrations above 1.8 mM is correlated with a decrease of V by about 60% and a continuous increase of K_m (Fig. 3). The final value for this parameters is about 166 μM compared to 100 μM at 3.3 mM Mg^{2+}.

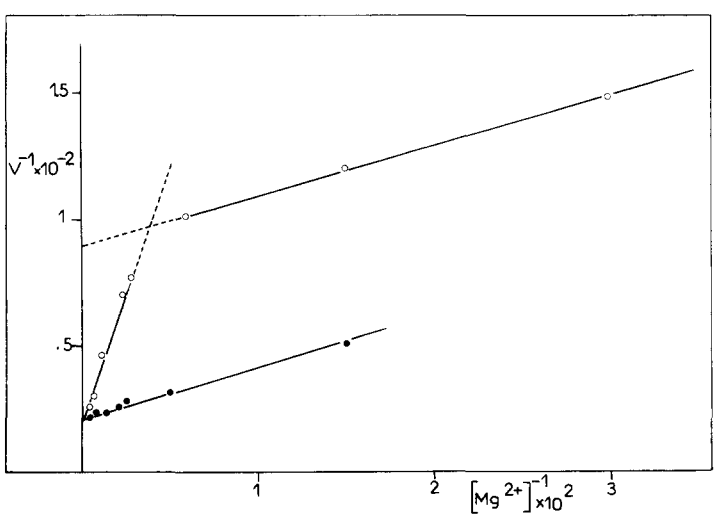

Fig. 4. The influence of Mg^{2+} on the activity of the isomerase in the proteolipidic particles. Reciprocal initial velocity measured with 5-androstenedione as substrate, is plotted versus reciprocal Mg^{2+} concentration. In these experiments, the EDTA concentration was either 3.3 mM (○), or no EDTA was added (●). Experimental conditions as in Fig. 3.

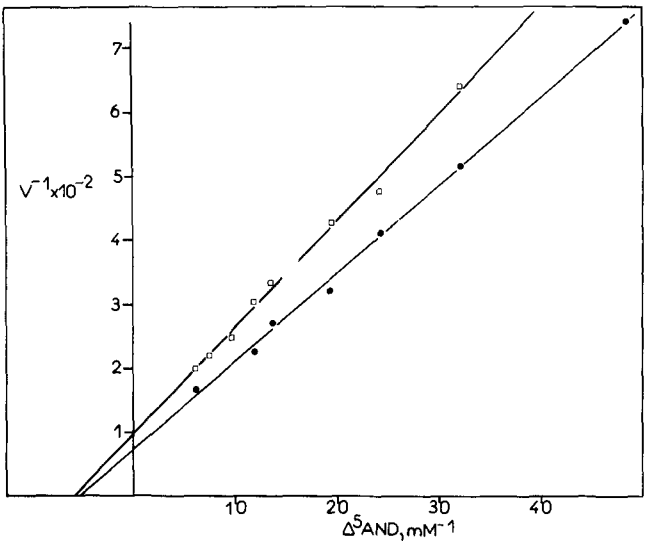

Fig. 5. Effect of Mg^{2+} on the isomerase in the proteolipidic particles treated with 3.3 mM EDTA. Reciprocal initial velocity is plotted versus reciprocal 5-androstenedione concentration. In these experiments, the concentration of Mg^{2+} was either 8.3 mM (•), or no ion was added (□). Experimental conditions as described in Materials and Methods.

(2) Reversibility of the inhibition. Addition in the assay buffer of increasing concentrations of $MgCl_2$ to the enzymatic dilution treated by 3.3 mM EDTA, leads to a composite activation (Fig. 2b). The reciprocal plots of V_i versus Mg^{2+} concentration show two straight lines. Their respective intersection with the ordinate gives the maximum activation for each part of the effect (Fig. 4). The first part of the effect fits with an increase of V as shown in Fig. 5. The second part occurs at greater ionic concentrations; this results in a

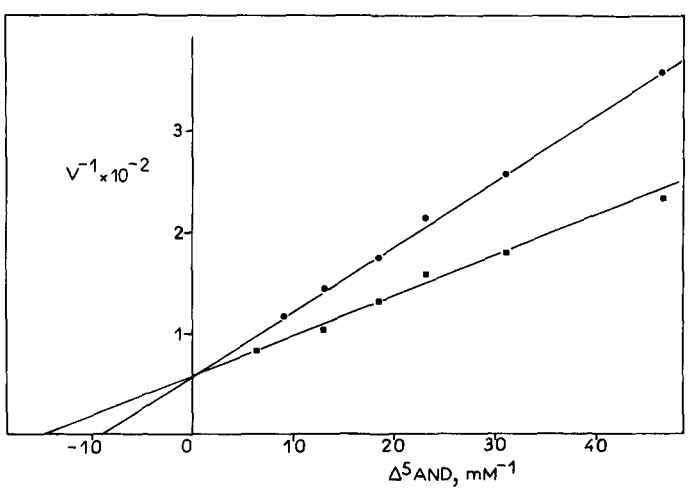

Fig. 6. Effect of Mg^{2+} on the isomerase in the proteolipidic particles in absence of EDTA. Reciprocal initial velocity is plotted versus reciprocal 5-androstenedione concentration. In these experiments, Mg^{2+} concentrations were either 4.6 mM (•), or 30 mM (■). Experimental conditions as described in Materials and Methods.

marked decrease of the substrate solubility, making impossible an accurate determination of K_m and V. The maximum amplitude of this second effect is 1.8 times the basic enzymatic activity measured in presence of 3.3 mM Mg^{2+} without EDTA in the assay medium.

Similarly, addition of $MgCl_2$ with no EDTA in the assay medium results in an increase of the initial enzymatic velocity. At the opposite of the preceding effect, reciprocal plot of V_i versus $MgCl_2$ concentration gives only one straight line. The maximum activation is again 1.8 times the initial reference velocity (Fig. 4). This activation is correlated with an increase of the substrate affinity as reflected by the decrease of K_m shown in Fig. 6. The mid point effect is at about 8 mM Mg^{2+}. It is observed that at these Mg^{2+} concentrations, the substrate affinity is higher in the proteolipidic particles (K_m = 60 μM in presence of 30 mM Mg^{2+}) than in the microsomes (K_m = 100 μM).

Discussion

It has been observed that monovalent salts (NaCl, CsCl, NaI) have no effect on the kinetic properties of the isomerase. A slight inhibition appears with KCl (Gallay, J., Vincent, M. and Alfsen, A., unpublished). Thus the activation due to a V increase of the isomerase appears to be specific of the divalent cations. The Cl^- being used with mono- and divalent cations the possible role of the anion can be neglected.

The accessibility of the isomerase to water and ions has been demonstrated by fluorescence technique. In addition, it is worthwhile to remember that the fluorescence polarization of equilenine, a competitive inhibitor of the isomerase, undergoes very slight changes in the Mg^{2+} concentration range effective for the activation effect, suggesting that no striking modification of the binding site conformation occurs in presence of low Mg^{2+} concentration [1]. On the other hand, the maximum activation extrapolated at infinite ionic concentration, could be related to the charge density and hydration energy of the different ions and is the highest with Mg^{2+} [9]. All the three cations have an ordering effect on the surrounding water [10—12]. The fact that proton transfers are faster in structured water than in a less organized aqueous medium is in agreement with this interpretation [13]. This hypothesis is further supported by the recent studies performed on the mechanism of isomerization of deuterated substrates by the microsomal isomerase [14]. In contrast with the experiments carried out with the bacterial enzyme, an exchange of deuterium with water protons is evidenced, suggesting the participation of water molecules to the proton transfer [14,15].

The V effect of divalent cations have been observed in the two systems under study, i.e. the microsomal isomerase and the "solubilized" particles. However, the proteolipidic particles present some specific features as well in their kinetic behavior as in their structural characteristics described by fluorescence measurements. These data clearly state that Mg^{2+} perturbs (directly or via the phospholipidic environment) the binding site of the enzyme which becomes more accessible to the substrate in presence of high ionic concentration in the proteolipidic particles. A tentative hypothesis might be that in the membranes the isomerase is located near ionic transport channels which could be probably the initiation centers of disruption of the microsomes induced by the ions [1].

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