Biochimica et Biophysica Acta 806 (1985) 35–41
Elsevier

BBA 41678

α,β-Bidentate CrADP abolishes the negative cooperativity of yeast mitochondrial F_1-ATPase

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(Received June 1st, 1984)

Key words: F_1-ATPase kinetics; Chromium-nucleotide complex; (Yeast mitochondria)

The hydrolysis of MgATP and MgITP by mitochondrial F_1-ATPase from *Saccharomyces cerevisiae* is competitively inhibited by α,β-CrADP, α,β,γ-CrATP and β,γ-CrATP. The apparent K_I values of the three complexes are in the range of the half-saturating MgATP concentration. The negative cooperativity ($n_H = 0.7$) of MgATP hydrolysis is totally abolished by α,β-CrADP ($n_H = 1.0$), while it is not affected by the CrATP. It is concluded that α,β-CrADP binds exclusively at the regulatory site and that CrATP binds exclusively to the catalytic site.

Introduction

The stable complexes of chromium (III) with ATP, ADP and several other nucleotides, first synthesized in Cleland's laboratory [1], have been used by a number of investigators (for references, see Refs. 2 and 3) either as inhibitors to elucidate kinetic mechanisms or as substrates to clarify the stereochemistry of enzymatic reactions. In the latter case use is made of the facts that these complexes exist as coordination isomers (mono-, bi- or tridentate) and as screw sense isomers (e.g., the tridentate α,β,γ-CrATP forms four isomers Λ and Δ, each as endo and exo), and that the pure diastereomers can be separated (for nomenclature and definitions, see Refs. 2 and 3).

These types of investigation were also performed with isolated H^+-ATPases from bovine heart mitochondria [4–6], rat liver mitochondria [4] and spinach chloroplasts [7] and with submitochondrial particles from bovine heart [6]. As far as the chromium complexes were applied as substrates [5–7] the results are not really conclusive and are partially controversial. As kinetic effectors, different isomers of CrATP * and CrADP were found to be all competitive inhibitors when MgATP was the substrate [4,6,7] and to be noncompetitive inhibitors with MgITP as the substrate [4,6]. In all cases, the negative cooperativity of MgATP [8–10] was not affected by these inhibitors [4,6]. In contrast, we demonstrate here that α,β-CrADP abolishes the negative cooperativity of F_1-ATPase from yeast mitochondria, while β,γ- and α,β,γ-CrATP do not. In addition, these three complexes are competitive inhibitors with respect to both MgATP and MgITP [11]. Finally, the effects of the diastereomeric Λ- and Δ-CrADP, separated by a new HPLC technique, are described.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; CD, circular dichroism.

* In these octahedral chromium complexes only those ligands are specified that are not water; α,β,γ indicate the coordinated phosphate oxygens. If not indicated by Λ or Δ the complexes were used as their mixtures of diastereomers (see Refs. 2 and 3).

Materials and Methods

All chemicals used were p.a. grade and purchased from E. Merck, Darmstadt. Pyruvate kinase, lactate dehydrogenase (both in 50% glycerol), ATP, ADP, ITP, NADH and phospho*enol*pyruvate were bought from Boehringer GmbH, Mannheim.

Yeast mitochondrial F_1-ATPase with a specific activity of 160 U/mg at pH 7.0 (about 190 U/mg at pH 8.0) was prepared from *Saccharomyces cerevisiae* by chloroform extraction as described elsewhere [10]. The enzyme was stored as an ammonium sulfate suspension and prior to use diluted with a 1 : 1 glycerol/water mixture containing 0.25 M sorbit, 50 mM Tris-HCl, 50 mM NaCl and 2 mM EDTA adjusted to pH 8.0. To remove the ammonium sulfate, 1 ml was centrifuged through a 5 ml syringe with Sephadex G-25 equilibrated with the same buffer. Bidentate β,γ-CrATP *, tridentate α,β,γ-CrATP and bidentate α,β-CrADP were prepared by the heating procedure described in Ref. 1. However, the purification by Dowex 50X2 chromatography was modified: since the ion-exchange resin catalyses the conversion of bidentate to tridentate CrATP, only small amounts of the reaction mixture were absorbed, and the β,γ-CrATP was eluted as soon as possible with 0.1 M sodium acetate (pH 4.75). After leaving the reaction mixture on the resin for 2 days, pure α,β-γ-CrATP was obtained. When α,β-CrADP was used as a mixture of diastereomers it was also eluted with 0.1 M sodium acetate (pH 4.75). In all cases, the fractions were immediately adjusted to pH 3 to prevent decomposition and polymerisation [2].

To separate the two diastereomers of α,β-CrADP the material was eluted from a Dowex 50X2 resin with 1 M $HClO_4$. The fractions were adjusted to pH 3.0 with solid $KHCO_3$, and the $KClO_4$ was filtered off. Separation was performed by HPLC on a C18 reverse-phase column using as elutant 0.05 M sodium phosphate (pH 5.0). From CD spectra the Λ-isomer eluted first, followed by the Δ-isomer. The fractions from a series of runs were immediately adjusted to pH 2.5, adsorbed on small Dowex 50X2 columns, eluted with 0.1 M sodium acetate (pH 4.75), and adjusted to pH 3. Under these conditions both isomers are stable for several months. (During the preparation of this manuscript a similar method was published [12], using ethanesulfonic acid instead of phosphate.) $Co(NH_3)_4ATP$ and $Co(NH_3)_4ADP$ were prepared according to Ref. 13 and purified on Dowex 50X2 with 0.1 M sodium acetate (pH 5.2) or 0.1 M Mes (pH 6.0), respectively.

All kinetic measurements were performed spectrophotometrically at 25°C. The final volume of the cuvette (2.02 ml) was made up of 0.2 M Hepes, adjusted to pH 7.0 with KOH, 4 mM phospho*enol*pyruvate, 0.5 mM NADH, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase and the appropriate concentrations of $MgCl_2$, ATP or ITP. Using a dissociation constant of 0.13 mM at pH 7.0 for both magnesium complexes [14], the total amounts of Mg^{2+} and ATP or ITP were adjusted to yield the desired concentrations of the substrates (i.e., MgATP or MgITP) and 1.0 mM free Mg^{2+}. For inhibition studies the appropriate concentrations of the chromium complexes were added. The reaction was started with 20 μl of the ATPase solution described above. At the concentrations used the chromium complexes had no effect on the coupled assay system. Decomposition at pH 7 was negligible during the period of measurement.

Initial velocities v were determined at a constant concentration of inhibitor [I], the substrate concentration [S] being varied from 15 μM to 5 mM MgATP or 50 μM to 2 mM MgITP. Data was analysed using the Hill equation according to the method of Wieker et al. [15] evaluating the maximal velocity Vmax, the interaction coefficient n_H and the substrate concentration $[S]_{0.5}$ giving $\frac{1}{2}V$max. In a second series of experiments, v was determined at constant [S] and variable [I]. From these data, fractional inhibitions $\eta = (v_0 - v)/v_0$ were calculated, and n_H, the maximal inhibition η_{max} and the inhibitor concentration $[I]_{0.5}$ giving $\frac{1}{2}\eta_{max}$ were determined as above. In some cases these measurements were also analysed by use of the Dixon plot [16], i.e., $1/v$ against [I]. Nonlinear relationships are presented by spline approximations, i.e., a piecewise least-squares fit of cubic polynomials using subroutine VB05B of the Harwell subroutine library.

* See footnote on p. 35.

Results

The three chromium complexes investigated here are strong inhibitors of the hydrolysis of MgATP or MgITP by the F_1-ATPase from yeast mitochondria. The analysis of initial velocities v measured at constant [I] (four or five concentrations) and variable [MgATP] or [MgITP] clearly revealed that the inhibitors had no effect on the maximal velocity (i.e., Vmax), but decreased the affinity of the enzyme for both substrates, i.e., $[S]_{0.5}$ increased. In Fig. 1, $1/V$max is plotted versus [I], and it can be seen that in each series the data vary less than $\pm 8\%$ around their mean (differences between the series are due to different enzyme preparations). Fig. 2 demonstrates the striking effects of these inhibitors on $[MgATP]_{0.5}$ (a comparable plot was obtained for $[MgITP]_{0.5}$). Regardless of their effects on n_H (see below) and the mechanism involved, the three chromium complexes are classified as competitive inhibitors with respect to MgATP and MgITP. A similar conclusion was reached using the Dixon plot (data not shown).

The hydrolysis of MgATP is characterised by negative cooperativity quantified by an interaction coefficient of $n_H \simeq 0.7$. As can be seen from Fig. 3, β,γ- and α,β,γ-CrATP have no effect on n_H, while α,β-CrADP at concentrations of at least 0.3 mM totally abolishes the negative cooperativity of MgATP yielding an $n_H \simeq 1.0$. This surprising effect is further demonstrated in Fig. 4. In contrast, the hydrolysis of MgITP is non-cooperative ($n_H = 1.0$) and the three inhibitors exert only a slight

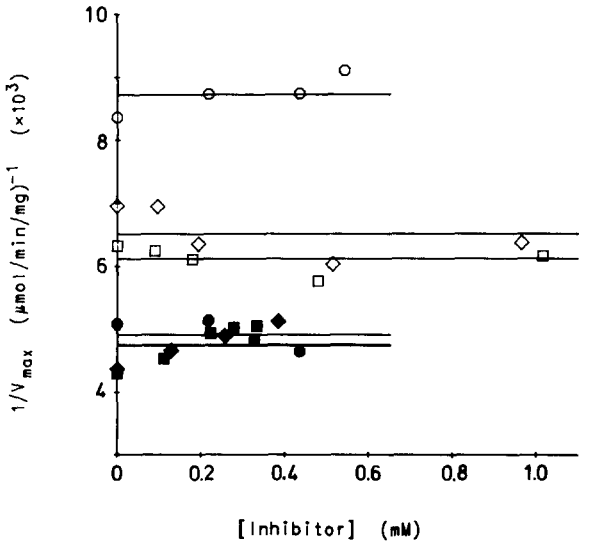

Fig. 1. Reciprocal maximum velocities $1/V$max as a function of [I]. Vmax was determined from v vs. [MgATP] (open symbols) or v vs. [MgITP] (closed symbols). The inhibitors were α,β-CrADP (○, ●), α,β,γ-CrATP (□, ■) and β,γ-CrATP (◇, ◆), respectively.

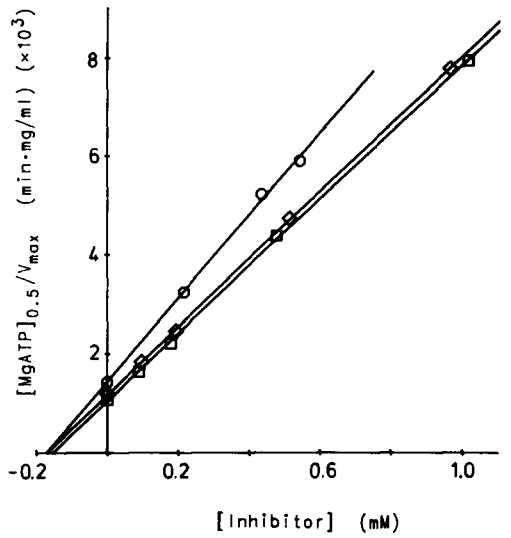

Fig. 2. Linear least-squares fits of $[MgATP]_{0.5}/V$max as a function of [I]. The intercepts on the abscissa give $-K_I$, the apparent inhibition constants. Symbols as in Fig. 1.

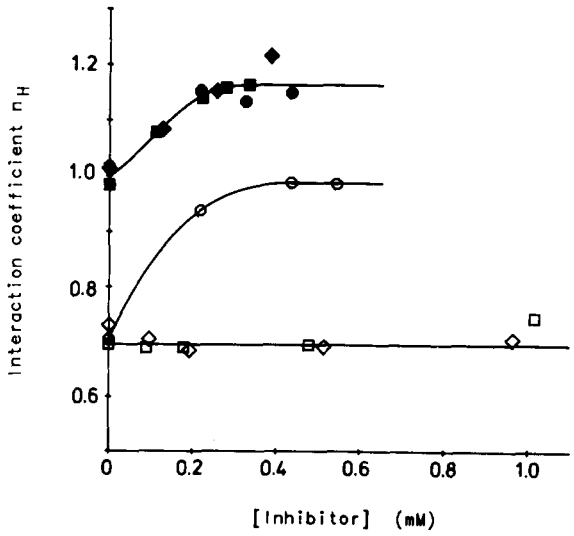

Fig. 3. Interaction coefficient n_H as a function of [I]. n_H was determined from v vs. [S]. Symbols as in Fig. 1.

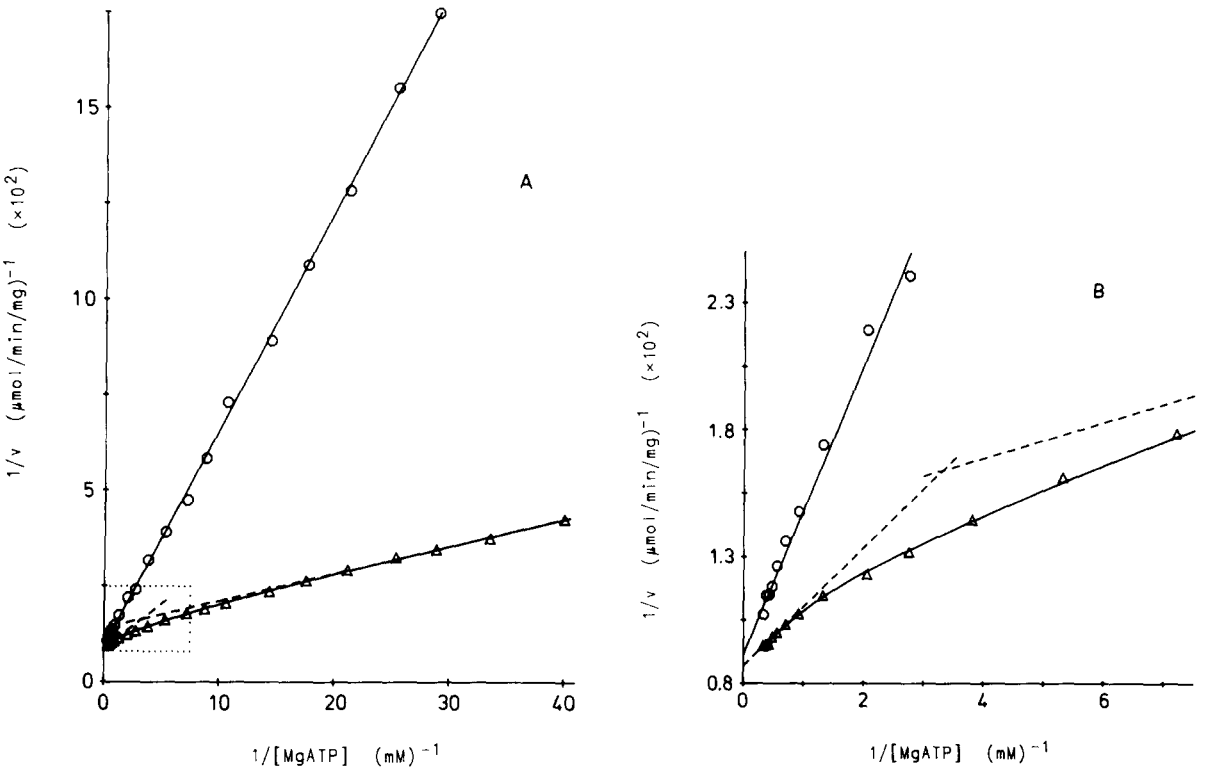

Fig. 4. Double-reciprocal plot of $1/v$ vs. $1/[MgATP]$. Initial velocities were measured in the absence (△) and presence (○) of 0.54 mM α,β-CrADP. The straight line (○——○) is a linear least-squares fit of all data, the dashed lines are least-squares fits of the six lowest and the six highest $1/[S]$ values, respectively, and the full line (△——△) is a spline fit of all data. The dotted area in (A) is magnified in (B).

effect on the interaction coefficient reaching a maximal value of $n_H \leqslant 1.16$.

Using MgATP as the substrate, the apparent inhibition constant (K_I) for each inhibitor was determined by linear least-squares fits of $[S]_{0.5}/V$max vs. [I] as shown in Fig. 2. (Since the determination of $[S]_{0.5}$ is affected by the error of Vmax we recommend a fit of $[S]_{0.5}/V$max leading to the same K_I, however, with greater accuracy.) The resulting K_I values are given in Table I to-

TABLE I

APPARENT INHIBITOR CONSTANTS K_I OF YEAST MITOCHONDRIAL F_1-ATPase

The K_I values (±S.D.) are determined by linear least-squares fits of $[MgATP]_{0.5}/V$max vs. [I] (Fig. 2) or $[I]_{0.5}$ vs. [MgITP] (Fig. 5), respectively.

Inhibitor	Substrate			
	K_I (mM)		K_S [a] (mM)	
	MgATP	MgITP	MgATP	MgITP
β,γ-CrATP	0.17±0.003	0.27±0.027	–	–
α,β,γ-CrATP	0.15±0.005	0.18±0.014	–	–
α,β-CrATP	0.17±0.013	0.07±0.003	–	–
None	–	–	0.17±0.003	0.48±0.007

[a] The apparent substrate constants K_S are the mean values of several $[MgATP]_{0.5}$ or $[MgITP]_{0.5}$, respectively, obtained in the absence of inhibitors.

gether with the apparent K_S value, the mean of the $[S]_{0.5}$ obtained in the absence of inhibitors. It is obvious that the affinity of F_1-ATPase to the three chromium complexes is quite the same as to MgATP.

Using MgITP as the substrate, initial velocities were also determined at constant [MgITP] and variable [I]. From these data fractional inhibitions η were calculated (see Methods) and analysed as functions of [I]. From this, values for n_H, $[I]_{0.5}$ and ηmax were determined [15]. In Fig. 5 the resulting $[I]_{0.5}$ are plotted vs. [MgITP], and from a linear least-squares fit the apparent K_I values obtained (Table I). From studies using CrATP values for n_H between 0.93 and 1.13 were obtained, the Dixon plots being linear. Using α,β-CrADP, the Dixon plots were concave upward (Fig. 6), and the n_H increased with [MgITP] up to $n_H \simeq 1.5$ at [MgITP] $\geqslant 0.5$ mM. These latter findings point to a positive cooperativity between α,β-CrADP and MgITP.

In additional experiments it was evident that the two diastereomers of α,β-CrADP exert similar effects. The Dixon plots (not shown) obtained with the isolated Λ- and Δ-isomers at four constant [MgATP] revealed that the K_I values differ only by a factor of 2, the Δ-CrADP being slightly more effective than the Λ-CrADP. Both isomers

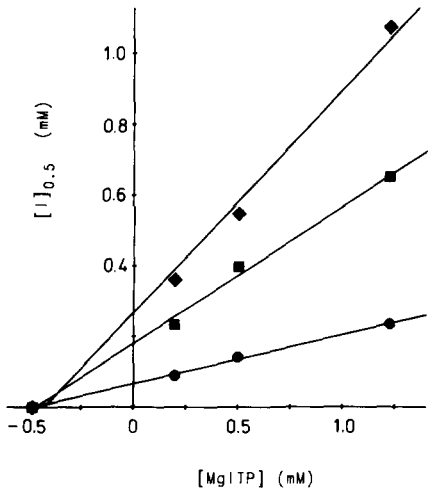

Fig. 5. Linear least-squares fits of $[I]_{0.5}$ as a function of [MgITP]. The $[I]_{0.5}$ were determined from η vs. [I]. The value on the abscissa, i.e., $[MgITP] = -K_S$ (see Table I), is part of each data set. The intercepts on the ordinate give K_I. Symbols as in Fig. 1.

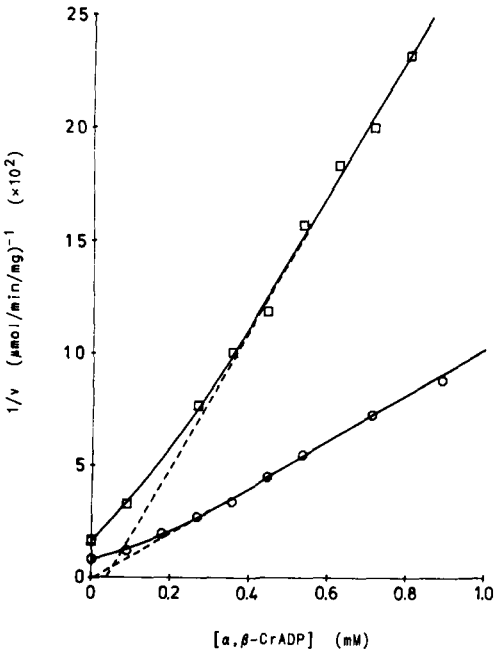

Fig. 6. Dixon-plot $1/v$ vs. [α,β-CrADP]. Measurements were performed in the presence of 0.2 mM (□) and 0.5 mM (○) MgITP. The full lines are spline fits of all data, the dashed lines are least-squares fits of the highest [I] values.

abolish the negative cooperativity of MgATP. With MgITP as substrate, the same ratio of K_I was found.

In addition to these experiments $Co(NH_3)_4$-ADP and $Co(NH_3)_4$ATP were also investigated; neither complex had any effect on the hydrolysis of MgATP and MgITP.

Discussion

In previous investigations on the structure and subunit composition, on the catalytic process and on the regulatory properties of F_1-ATPases from different sources, no significant differences were found. However, the effects of the chromium nucleotide complexes on yeast mitochondrial F_1-ATPase are significantly different from their effects on other F_1-ATPases [4,6] (see Introduction). The present results, in particular the influence on the negative cooperativity of MgATP hydrolysis, suggest that the yeast enzyme has a greater binding specificity than other F_1-ATPases. Since the overall processes are very similar, these differences

in the details of function and regulation are accessible only from kinetic studies. Therefore, a mechanistic interpretation of our results and the characterization of binding sites have to be based on kinetic considerations.

The kinetic investigations of F_1-ATPases from different sources clearly revealed at least two different types of nucleotide binding site, the catalytic sites (C-sites) allosterically interacting with the regulatory sites (R-sites) [17,18] (for references, see also Ref. 19). Recently, a mechanism for yeast mitochondrial F_1-ATPase was proposed by our group [17] which accounts for the negative cooperativity of ATP hydrolysis as well as for the allosteric effects of different anions: the enzyme with its $\alpha_3\beta_3\gamma\delta\varepsilon$ subunit structure contains three noninteracting protomers, each possessing one C-site allosterically interacting with one R-site. MgATP is bound at the C-site and the R-site, both being in a high-affinity state; binding of MgATP or some anions [17] at the R-site switches the C-site to its low-affinity state, which consequently leads to negative cooperativity. In contrast, MgITP binds only at the C-site and consequently exhibits no cooperative effects. As a consequence of microscopic reversibility, binding of substrates at the C-site has to switch the R-site from a high-affinity to a low-affinity state.

The experimental results can be compared with the predictions based on this mechanism for the three possibilities of inhibitor binding: (1) the inhibitor is exclusively bound at the C-site; (2) the inhibitor is exclusively bound at the R-site; (3) the inhibitor is bound at both the C- and the R-site.

Case (1). If the inhibitor is exclusively bound at the C-site it competes directly with the substrate, and a competitive type of inhibition will result. Although the inhibitor induces the low-affinity state of the R-site, MgATP is the only ligand bound at the R-site and regulating the binding of substrate at the C-site. Therefore the negative cooperativity of MgATP will not be affected by the inhibitor. These predictions are in contrast to the results obtained with α,β-CrADP (Fig. 3), but they fit exactly the results with β,γ-CrATP and α,β,γ-CrATP.

Case (2). If the inhibitor is exclusively bound at the R-site, it competes with MgATP and inhibits the binding of both substrates at the C-site by inducing the low-affinity state. Since only binding and not the catalytic process is altered, a competitive type of inhibition will be observed with MgATP and MgITP. Regardless of whether the inhibitor or MgATP is bound at the R-site, the C-site will be in its low-affinity state. Therefore, at sufficiently high [I] the negative cooperativity of v vs. [MgATP] will be abolished. If the inhibition is measured at constant [S] as a function of [I], the R-site will be in its low-affinity state due to the substrate bound at the C-site. In the case of MgITP, the inhibitor will displace the substrate by induction of the low-affinity state of the C-site, and the resulting dissociation of MgITP leads to the high-affinity state of the R-site. However, with MgATP both substrate and inhibitor compete for the R-site and the affinity of the C-site will not change. Therefore, the inhibition measured at constant [S] as a function of [I] will exhibit positive cooperativity with respect to MgITP as the substrate and be noncooperative with MgATP. These predictions are in contrast to the results obtained with β,γ-CrATP and α,β,γ-CrATP. However, they fit exactly the results obtained with α,β-CrADP: in case of MgITP being the substrate $n_H \simeq 1.5$ is evaluated from η vs. [I] and $1/v$ vs. [I] is found to be concave upward (Fig. 6). When MgATP is the substrate an $n_H = 1$ is obtained from v vs. [MgATP] (Fig. 3) and $1/v$ vs. [I] is found to be linear.

Case (3). If the inhibitor is bound at both sites, competitive inhibition will be observed and the negative cooperativity will be abolished for the reasons outlined above. However, the competition of MgITP and the inhibitor at the C-site will prevent any cooperativity of η vs. [I]. These predictions do not fit the results obtained with either β,γ-CrATP and α,β,γ-CrATP nor those obtained with α,β-CrADP.

In summary, the considerations lead to the conclusion that β,γ-CrATP and α,β,γ-CrATP are exclusively bound at the C-site and that α,β-CrADP is exclusively bound at the R-site. This latter finding is surprising, since ADP is a substrate of ATP synthesis and would therefore be expected to bind at the C-site. Possibly, binding of ADP necessitates the presence of phosphate, but since phosphate also inhibits the enzyme, it cannot be determined whether it induces an additional binding of α,β-CrADP at the C-site. Conversely, the 'hys-

teretic inhibition' by ADP, described for F_1-ATPase from pig-heart mitochondria [19], demonstrates a highly selective regulatory function of the ADP moiety, which correlates with our interpretation. Unfortunately, the instability of the chromium complexes do not permit the long-time incubations necessary to study whether α,β-CrADP exhibits 'hysteric inhibition'.

Although the binding sites are highly selective for either CrATP or CrADP, there is no significant selectivity for the sterical arrangement of the chromium complexes: the C-site has the same affinity for β,γ-CrATP with its monocyclic coordination at the metal ion as for α,β,γ-CrATP which has a bicyclic coordination. The affinity of the R-site for Λ- and Δ-CrADP differs only by a factor of 2, much lower than expected for selectivity. However, the metal nucleotide complexes are not bound at any site, if the metal-coordinated water is replaced by ammonia, i.e., neither $Co(NH_3)_4$ ATP nor $Co(NH_3)_4$ADP is an inhibitor of yeast mitochondrial F_1-ATPase. A possible explanation may be that the metal ion is bound to an enzyme residue via a hydrogen bond of the coordinated water, which is much more acidic than the ammonia [2]. However, the opposite explanation may also hold, i.e., the binding process requires the replacement of water by an enzyme residue, which cannot occur in the case of the unexchangeable ammonia.

Acknowledgements

We thank Mr. K.-H. Wüster for the preparation of the enzyme and the metal nucleotide complexes, Mrs. A. Bremer for the kinetic measurements, Dr. Th. Plesser for helpful discussion and Dr. L. Smart for reading the English content of the manuscript.

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