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Action of hexaamminecobalt on the activity of *Serratia marcescens* nuclease

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Abstract

Using CD spectroscopic and kinetic analysis, a refined mechanism of $Co(NH_3)_6^{3+}$ action on activity of *Serratia marcescens* nuclease was elucidated. The mechanism was identical with previously found mechanisms of Mg^{2+} and $C_7H_5O_2Hg^+$. Similarly to Mg^{2+} and $C_7H_5O_2Hg^+$, $Co(NH_3)_6^{3+}$ binding to the DNA substrate induced changes in the secondary structure which resulted in changes of the enzymatic activity of the *S. marcescens* nuclease. Upon binding of 0.03 $Co(NH_3)_6^{3+}$ per DNA phosphate, highly polymerized DNA displayed A-form characteristics. The DNA transition from B-form to A-form intermediate was followed by a decrease of the nuclease activity. The diminishing nuclease activity was consistent with diminishing values of Km and Kcat. $Co(NH_3)_6^{3+}$ binding to the highly polymerized DNA caused a 1.7–2.8-fold decrease in Km, and 13.3–19.9 decrease in Vmax compared with Mg-DNA complex. A vast excess of $Co(NH_3)_6^{3+}$ did not affect the activity of *S. marcescens* nuclease if the DNA in the assay mixture remained in its B-form conformation. Preincubation of *S. marcescens* nuclease with $Co(NH_3)_6^{3+}$ did not influence the tertiary structure of the enzyme.

Introduction

The extracellular endonuclease of the Gram negative bacterium *Serratia marcescens* (EC 3.1.30.2), Sma nuc, potently degrades both DNA and RNA without a pronounced preference to the sugar moieties. It is a well studied enzyme of known structure (Biedermann *et al.* 1989; Miller *et al.* 1994), mechanism of action (Friedhoff *et al.* 1996) and physical-chemical and biochemical properties (Nestle & Roberts 1969; Filimonova *et al.* 1980, 1981; Kolmes *et al.* 1996).

Sma nuc is a unique enzyme. It represents a large group of homologous nucleases which are widely found in nature and share functionally important amino-acid residues (Friedhoff *et al.* 1996). Sma nuc is one and only prokaryotic endonuclease representing a family of isoforms. Two major isoforms of Sma nuc were isolated and characterized (Filimonova *et al.* 1991; Pedersen *et al.* 1993a, b; Suh *et al.* 1995; Filimonova *et al.* 1996, 1997, 1999). Sma nuc is a rare enzyme which is activated with the metal, Mg^{2+} cations, when Mg^{2+} cations change the substrate's conformation upon binding to it (Filimonova *et al.* 1997). Although the interaction of enzyme's effectors with its substrates is predicted as a type of regulation of enzymatic activity, regulation of this type is seldom noted with nucleases. Except for Sma nuc, only DNase I was noted to be regulated by metal binding (also Mg^{2+}) to its substrate (Poulos & Price 1972). However activity regulation of DNA binding enzymes by changing the local structure of DNA when the enzyme's effectors bind may play a significant role *in vivo*; such regulation may affect both the function and metabolism of the DNA segments in the immediate vicinity of the bound effectors and result in altered nucleic acids metabolism.

Besides Mg^{2+}, Sma nuc is regulated by Fe^{2+}, Ca^{2+}, Zn^{2+}. All these cations, except for Mg^{2+}, repress the nuclease activity (Leshchinskaya *et al.* 1967; Nestle & Roberts 1969). 4-(chloromercurio)benzoate ($C_7H_5O_2Hg^+$ in solution), a reagent specific for the SH-groups of proteins, also repressed the nuclease activity although Sma nuc lacks SH- groups (Filimonova *et al.* 1980; Pedersen *et al.* 1993b). The investigation revealed a similarity in the mechanism of action of $C_7H_5O_2Hg^+$ and Mg^{2+} on the nuclease activity (Filimonova *et al.* 2001). Both $C_7H_5O_2Hg^+$ and Mg^{2+} affected the nuclease activity when they had bound to the substrate (DNA), and as a result changed the substrate's secondary structure. The similarity between Mg^{2+} and $C_7H_5O_2Hg^+$ is that both contain a cation, although the metals are markedly different. From this premise, we hypothesized that the mechanism of most other metal-containing effectors towards the activity of Sma nuc is similar to the mechanism of Mg^{2+} and $C_7H_5O_2Hg^+$.

To examine this hypothesis, we studied $Co(NH_3)_6^{3+}$ as an effector of the nuclease activity. Although $Co(NH_3)_6^{3+}$ does not occur naturally in biological systems it is a well studied model cation that is known as a modulator of the polynucleotide structure. Binding to oligonucleotides of d(G-C) types, $Co(NH_3)_6^{3+}$ induces the oligonucleotide transformation from B- to A- form conformation (Xu *et al.* 1993). Nothing, however, is known of the $Co(NH_3)_6^{3+}$ action on activity of *S. marcescens* nuclease.

We studied the effect of $Co(NH_3)_6^{3+}$ on DNA and Sma nuc conformations as well as the nuclease activity. Experiments were undertaken with the isoform Sm2 which is the mature Sma nuc protein.

Materials and methods

Preparation of the nuclease

The nuclease was isolated from the fermentation broth of *S. marcescens* B10M1 and the isoforms were separated as described previously using anion- and cation-exchange chromatography on DEAE-cellulose DE-32, phosphocellulose P-11 and DEAE-cellulose DE-52 (Filimonova *et al.* 1980, 1991). The homogeneity of the enzyme preparations has been previously described (Pedersen *et al.* 1993b).

Sample preparation

To study $Co(NH_3)_6^{3+}$ influence on highly polymerized DNA (type XIV, Sigma), a DNA solution in 50 mM Tris-HCl buffer was titrated by adding microliter amounts of $Co(NH_3)_6Cl_3$ stock solution (0.14 M) to 0.03, 0.06 or 0.2 $Co(NH_3)_6^{3+}$ per DNA phosphate. Here and thereafter used Tris was preliminary recrystallized in the presence of EDTA to prevent possible contamination with trace amounts of multivalent metal cations.

When $Co(NH_3)_6^{3+}$ dependence was studied in the presence of Mg^{2+} the DNA solution was preliminarily mixed with 1 M $MgSO_4$ to 30 Mg^{2+} per DNA phosphate and then titrated with the appropriate amount of stock solution of $Co(NH_3)_6Cl_3$ to 0.03 or 0.2 $Co(NH_3)_6^{3+}$ per DNA phosphate.

Studying a direct influence of $Co(NH_3)_6^{3+}$ on *S. marcescens* nuclease, to remove the cations of multivalent metals from the Sm2 isoform preparation, a column chromatography (1 × 26.3 cm) on Sephadex G-75 (fine) equilibrated with 50 mM Tris-HCl buffer, pH 8.5, was performed as done previously (Poulos & Price 1972; Filimonova *et al.* 1997). Then 0.14 M $Co(NH_3)_6Cl_3$ was added to the Sm2 preparation to a ratio of $Co(NH_3)_6^{3+}$/Sm2 of 57.8, 288.8, or 577.6. After 15 min preincubation at room temperature the CD spectra of the Sm2 preparation in the presence or in the absence of $Co(NH_3)_6^{3+}$ were recorded. Enzymatic activity of these samples were assayed in a few hours later.

Circular dichroism spectrometry (CD)

CD spectra of 5.02 μM Sm2 or 1.44 mM (in a nucleotide equivalent) DNA were recorded at room temperature in a 10 mm or 2 mm path length cuvette at 210–310 nm using a Jasco-J 500 A spectrometer .

Kinetic study

The nuclease activity was determined by the hyperchromic effect of hydrolysis of the highly polymerized DNA using a λ-35 Perkin Elmer spectrophotometer. Apparent rates of the reaction were recorded until the progress curves became non-linear. Rates were calculated from the linear part of the reaction progress curves (initial velocities) using the applied Rate Analysis software package.

Experiments were carried out in 10 mm cuvettes at 25 °C. After addition of 0.51 μM isoform Sm2

(13.63 μg/ml) to 500-fold volume (2500 μl) of pre-warmed (3–5 min) assay mixture containing 50 mM Tris-HCl buffer, pH 8.55, and appropriately prepared DNA the measurements were performed for 5–30 min.

Activity of Sma nuc, pre-incubated with $Co(NH_3)_6^{3+}$, was determined at a DNA concentration of 0.03 mg/ml. To establish the crude Km value the substrate concentrations varied between 5 and 250 μg/ml. For fine kinetic measurements the DNA concentrations were 5–125 μg/ml.

Activity of *S. marcescens* nuclease was expressed in terms of Kunitz unit (KU). 1 KU is defined as the amount of enzyme needed for an increase of 0.001 nm/min at 25°C in a 1 ml volume at 10 mm path length.

The reaction velocities were expressed in KU per mg of Sma nuc in the assay mixture. Concentration of the isoform Sm2 was calculated based on the molecular mass and molar extinction coefficient of 47.292 M^{-1} cm−1 (Filimonova *et al.* 1981; Pedersen *et al.* 1993a). Concentration of the substrates in nucleotide equivalents were calculated using ε_{260} of 6500 M^{-1} cm^{-1}.

Km and Vmax values were determined from Lineweaver-Burk double reciprocal-, Eadie-Hofstee- and Hanes transformed Michaelis-Menten plot. The slope of the Lineweaver-Burk-, Eadie-Hofstee- or Hanes transformed plots gave Km/V_{max}, -Km or $1/V_{max}$, respectively. The value of ordinate axis vesus 0 at the abscissa axis gave $1/V_{max}$, V_{max} or Km/V_{max}, respectively. Kcat was calculated as V_{max} per 1 mM of Sma nuc.

Results and discussion

Action of hexaaminecobalt on *S. marcescens* nuclease activity was analyzed in comparison with the action of these cations on both the nuclease and DNA conformations noted via CD spectroscopic analysis. The effect of $Co(NH_3)_6^{3+}$ has been examined in comparison with that of Mg^{2+}.

CD spectra of DNA preparations are shown in Figure 1. As seen from the figure, the CD spectrum of commercial DNA (line 1) without addition of the cations (taken as a control) is the spectrum of B-form DNA (Hung *et al.* 1994); it exhibits large and small positive bands centered at 273 and 218 nm, respectively, a large negative band centered at 243 nm and intersections of the abscissa at 224 and 256 nm.

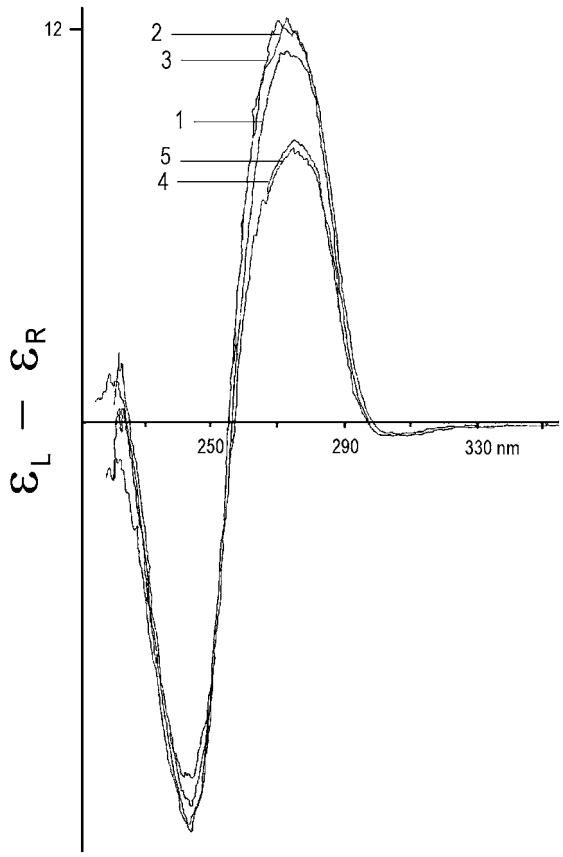

Fig. 1. CD spectra of DNA preparation in the absence (1) and in the presence (2-5) of $Co(NH_3)_6^{3+}$ at $Co(NH_3)_6^{3+}$ to DNA phosphate ratio of 0.03 (2,4) and 0.2 (3.5). The Mg^{2+} to DNA phosphate ratio (4,5) was 30.

Addition of $Co(NH_3)_6Cl_3$ to the DNA induced small but specific binding changes to the polynucleotides (Xu *et al.* 1993) as observed in the CD spectra (lines 2 and 3). The positive band at 255–300 nm broadened and the intensity of the positive band at 271 nm increased by 10%. These changes suggest the DNA transition to A-form characteristics due to $Co(NH_3)_6^{3+}$ binding. Alteration of the $Co(NH_3)_6^{3+}$ to DNA phosphate ratio (0.03 and 0.2) resulted in nearly superimposed spectra (spectra 2 and 3). The superimposing spectra allowed us to assume that saturation of the DNA with $Co(NH_3)_6^{3+}$ happens at rather low binding density of the cations (Braunlin & Xu 1992) that was about 0.03 $Co(NH_3)_6^{3+}$ per DNA phosphate. CD spectra of DNA preparations in the presence of Mg^{2+} together with $Co(NH_3)_6^{3+}$ (spectra 4 and 5) support the assumption. The spectra of these preparations also superimposed despite the noted difference in the ratio of $Co(NH_3)_6^{3+}$ to DNA phosphate. Spectra of prepa-

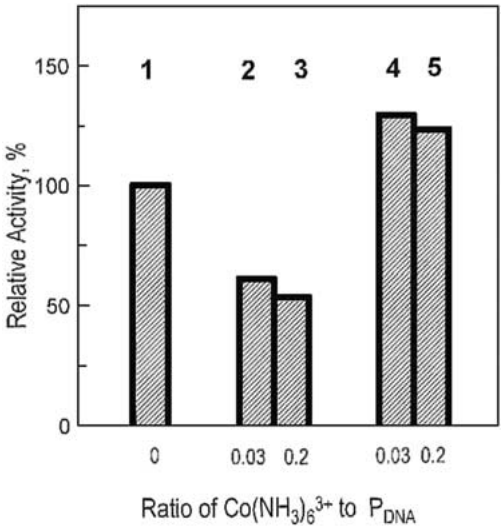

Fig. 2. The nuclease activity towards the DNA preparations demonstrating B-form (1,4,5) and A-form (2,3) characteristics. DNA preparation: 1- commercial, 2- 5 - with $Co(NH_3)_6Cl_3$, 4-5 - with $Co(NH_3)_6Cl_3$ and Mg^{2+}. Ratio of $Co(NH_3)_6^{3+}$ to DNA and Mg^{2+} to DNA are as noted in Figure 1.

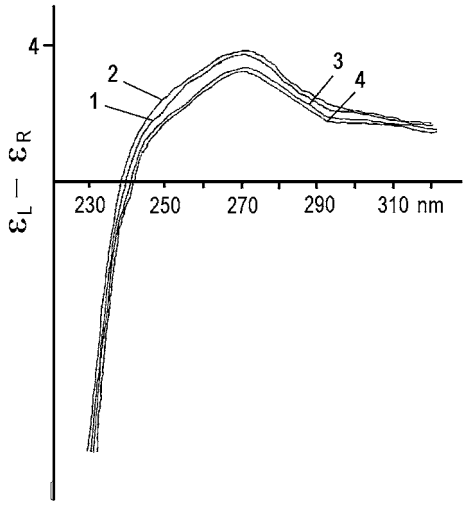

Fig. 3. CD spectra of Sma nuc preparation in the absence (1) and in the presence (2-4) of $Co(NH_3)_6^{3+}$ at $Co(NH_3)_6^{3+}$ to the nuclease ratio of 57.8 (2), 288.8 (3), and 577.6 (4).

rations containing Mg^{2+} (spectra 4 and 5), however, differed from the spectra of DNA in the presence of solely $Co(NH_3)_6^{3+}$ (spectra 2 and 3) by a pronounced diminution of the positive band centered at 275 nm and a narrowing of the positive band at 255–300 nm. The reduced intensity of the positive band evidences of the Mg^{2+} association with the phosphate groups of DNA and formation of Mg-DNA complex that results in changing of the secondary structure of DNA (Chan *et al.* 1979; Johnson *et al.* 1981; Watanabe & Iso 1984).

Enzymatic activity of Sma nuc towards the commercial DNA (1) and the $Co(NH_3)_6^{3+}$ (2,3) or $Co(NH_3)_6^{3+}$ together with Mg^{2+}(4,5) preparations is depicted in Figure 2. As noted in the figure, the nuclease activity on the Mg-DNA complex associated with $Co(NH_3)_6^{3+}$ was 20–30% higher and DNA solely associated with $Co(NH_3)_6^{3+}$ was 40–45% lower than the activity with commercial DNA (taken as 100%).

Since the complicity of $Co(NH_3)_6^{3+}$ association with DNA and the ratio of the bound to free DNA in solution with $Co(NH_3)_6^{3+}$ was not clear we examined the influence of $Co(NH_3)_6^{3+}$ on both the conformation and the activity of *S. marcescens* nuclease at a wide range of $Co(NH_3)_6^{3+}$/ Sma nuc ratio. Sma nuc was preincubated with $Co(NH_3)_6Cl_3$ at $Co(NH_3)_6^{3+}$ to Sma nuc ratios of 57.8, 288.8, and 577.6 and then was subjected to both CD spectroscopic and rate analyses. CD spectra of the Sma nuc preparations are shown in Figure 3. The CD spectrum of the nuclease in the absence of $Co(NH_3)_6^{3+}$ (1) superimposed with the spectrum of Sma nuc in the presence of 57.8-fold excess of $Co(NH_3)_6^{3+}$ (2) and was identical with the spectra recorded in the presence of $Co(NH_3)_6^{3+}$ at the higher concentrations (3 and 4). Preincubation of Sma nuc with $Co(NH_3)_6^{3+}$ thus did not affect the aromatic CD spectrum of Sma nuc indicating a lack of $Co(NH_3)_6^{3+}$ influence on the tertiary structure of *S. marcescens* nuclease. This conclusion was supported by results of the rate analysis shown in Figure 4. Nuclease preincubated with $Co(NH_3)_6^{3+}$, followed by being appropriately diluted, displayed similar activity towards the highly polymerized DNA when the range of $Co(NH_3)_6^{3+}$ to the nuclease ratio varied from 0 to 577.6. Statistical analysis showed that difference between the values of nuclease activity found at this wide range of $Co(NH_3)_6^{3+}$ concentration was insignificant. It is worthy to note that 20-fold dilution with the water and 500-fold dilution with the assay mixture did not influence the $Co(NH_3)_6^{3+}$ to Sma nuc ratios and resulted in the assay mixture 10-100 fold less than the amount of $Co(NH_3)_6^{3+}$ needed to induce the transition of DNA from B- to A-form characteristics. In addition, the assay mixture contained 30 Mg^{2+} per DNA phosphate that was enough to maintain DNA in its B-form conformation (Filimonova *et al.* 1997). A vast excess of $Co(NH_3)_6^{3+}$ thus did not affect the activity of Sma

Table 1. Kinetic parameters for the cleavage of DNA in the presence of the cations.

Estimation plot	Km, mg/ml		Vmax, mM/min		Kcat, sec^{-1}	
	$Co(NH_3)_6^{3+}$	Mg^{2+}	$Co(NH_3)_6^{3+}$	Mg^{2+}	$Co(NH_3)_6^{3+}$	Mg^{2+}
Lineweaver -Burk-	0.018	0.046	120.4	1993.1	53.7	888.2
Eadie- Hofstee-	0.007	0.017	98.0	1390.2	43.7	619.5
Hanes transformed						
Michaelis- Menten	0.018	0.031	120.4	1655.3	53.7	737.6

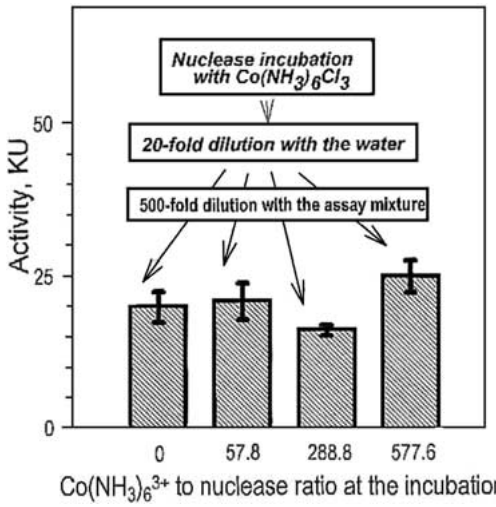

Fig. 4. Activity of the nuclease preincubated with Co(NH3)6Cl3 at $Co(NH_3)6Cl3$ to Sma nuc ratio of 0, 57.8, 288.8, and 577.6. At the assay mixture $Co(NH_3)6Cl3$ to DNA phosphate ratio was respectively 0 (control), 0.00003, 0.0015, and 0.003; Mg^{2+} to DNA phosphate ratio was 30; Mg^{2+} to the nuclease ratio - 5×10^6.

nuc when the substrate in the assay mixture remained in the B-form conformation.

Kinetic analysis was used to refine the effect of $Co(NH_3)_6^{3+}$ action on the catalytic mechanism of DNA cleavage with *S. marcescens* nuclease compared with that of Mg^{2+}. Three different plots were used to insure reliability in the determination of kinetic parameters (Figure 5). While the estimations depended on the particular method used, similar estimations of the kinetic parameters when $Co(NH_3)_6^{3+}$ was present were found. $Co(NH_3)_6^{3+}$ binding to the highly polymerized DNA caused a 1.7–2.8-fold decrease in Km, and a 13.3–19.9 decrease in Vmax compared with Mg-DNA complex (Table 1). The association thus of $Co(NH_3)_6^{3+}$ with DNA such that a DNA-transition to A-form characteristics occurred, resulted in a moderately enhanced affinity of the *S. marcescens* nuclease to the transformed substrate and drastically reduced a productive dissociation of the enzyme-substrate complex.

Conclusion

Using $Co(NH_3)_6Cl_3$ the transition of highly polymerized DNA to A-form characteristics was induced as revealed by CD-spectroscopy. The transition was followed by a decrease of the nuclease activity. Therefore $Co(NH_3)_6^{3+}$ influenced enzymatic activity of *S. marcescens* nuclease. The diminished nuclease activity was consistent with diminished values of Km and Kcat, but the Km value decreased only moderately while the Kcat value changed drastically. $Co(NH_3)_6^{3+}$ binding to DNA thus mainly influenced the catalytic function of *S. marcescens* nuclease and to a lesser extent the enzyme's affinity to the substrate. The mechanism of $Co(NH_3)_6^{3+}$ action on activity of *S. marcescens* nuclease was identical with previously found mechanisms of Mg^{2+} and $C_7H_5O_2Hg^+$. Similar to Mg^{2+} and $C_7H_5O_2Hg^+$ $Co(NH_3)_6^{3+}$ binding to the substrate, DNA, induced changes in the secondary structure of the substrate that were responsible for the altered enzymatic activity of the *S. marcescens* nuclease. This finding is in agreement with previous studies that have noted *S. marcescens* nuclease is sensitive to the secondary structure of its substrates.

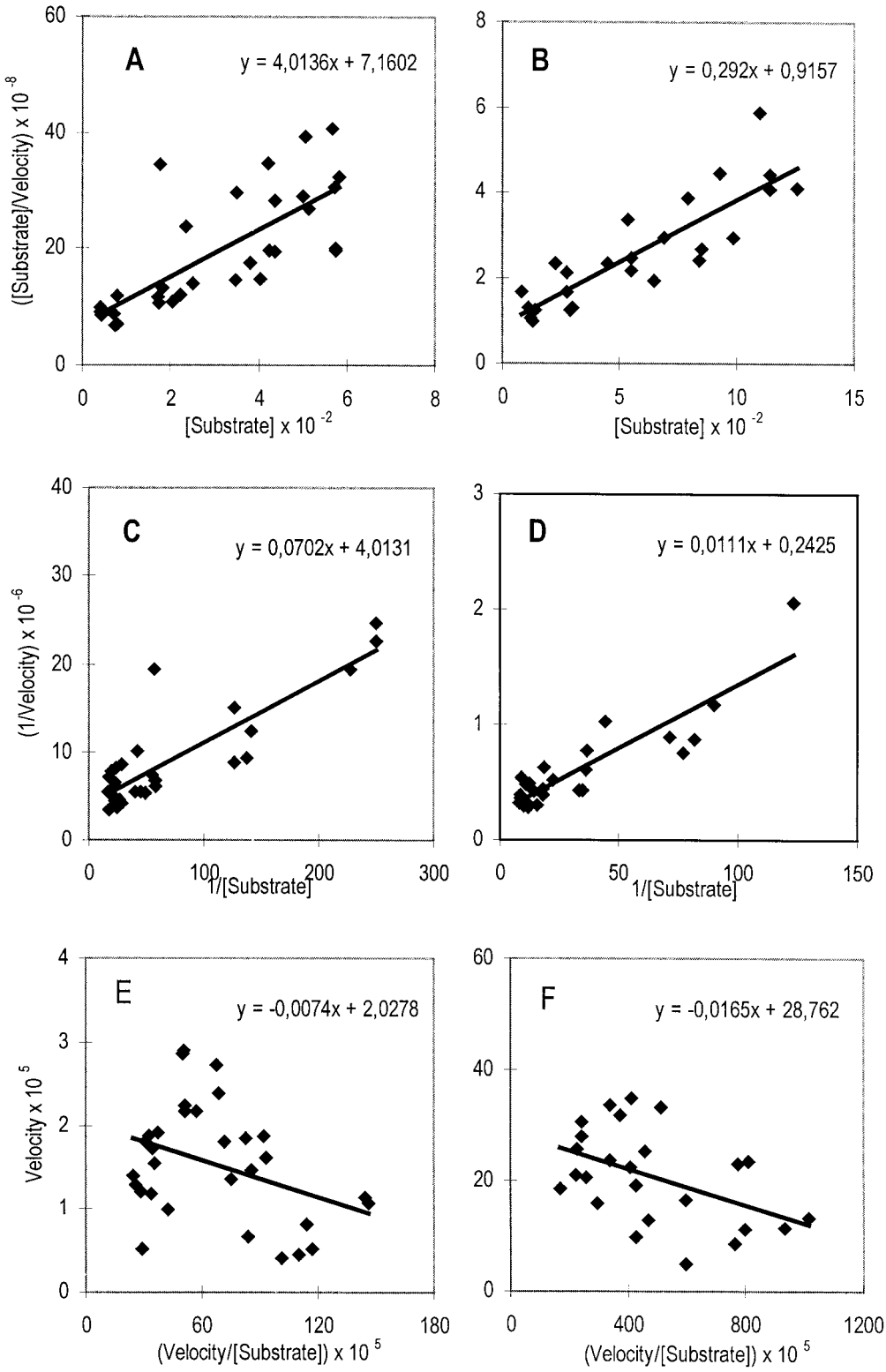

Fig. 5. Estimation of the kinetic parameters from Hanes transformed Michaelis-Menten - (A, B), Lineweaver-Burk - (C, D) and Eadie-Hofstee plots (E, F). DNA was incubated with 0.06 $Co(NH_3)_6^{3+}$ (A, C, E) or 30 Mg^{2+} (B,D,F) per DNA phosphate.

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