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Metal ion binding to D-xylose isomerase from *Streptomyces violaceoruber*

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The binding of two activating cations, Co^{2+} and Mg^{2+}, and of one inhibitory cation, Ca^{2+}, to D-xylose isomerase from *Streptomyces violaceoruber* was investigated. Equilibrium-dialysis and spectrometric studies revealed that the enzyme binds 2 mol of Co^{2+}/mol of monomer. Difference absorption spectrometry in the u.v. and visible regions indicated that the environment of the first Co^{2+} ion is markedly different from that of the second Co^{2+} ion. The first Co^{2+} appears to have a six-co-ordinate octahedral symmetry, whereas the symmetry of the second Co^{2+} is less evident, being four- or five-co-ordinate. The conformational change induced by binding of Co^{2+} to the first site is maximum after the addition of 1 equivalent of Co^{2+} and yields a binding constant $\geqslant 3.3 \times 10^6$ M^{-1}. Binding of Co^{2+} to the second, weaker-binding, site caused a visible difference spectrum. The association constant estimated from Co^{2+} titrations at 585 nm agrees satisfactorily with the value of 4×10^4 M^{-1} obtained from equilibrium dialysis. Similarly, the enzyme undergoes a conformational change on binding of Mg^{2+} or Ca^{2+}, the binding constants being estimated as 1×10^5 M^{-1} and 5×10^5 M^{-1} respectively. Competition between the activating Mg^{2+} and Co^{2+} and the inhibitory Ca^{2+} ion for both sites was further evidenced by equilibrium dialysis and by spectral displacement studies.

INTRODUCTION

D-Xylose isomerases (EC 5.3.1.5) catalyse the conversion of D-xylose and D-glucose into D-xylulose and D-fructose respectively, and have attracted considerable interest for the production of high-fructose corn syrup and ethanol.

D-Xylose isomerases are dependent on the bivalent cations Mg^{2+}, Co^{2+} or Mn^{2+} for catalytic activity. These metal ions activate D-xylose isomerases to different extents, depending on the origin of the enzyme and the substrate (Chen, 1980). In the specific case of D-xylose isomerase from *Streptomyces violaceoruber*, the highest activity towards D-xylose and D-glucose is obtained with Mg^{2+}, although Co^{2+} and Mn^{2+} support activity with various degrees of efficiency (Callens *et al.*, 1986). Furthermore, stabilization of D-xylose isomerase by metal ions has been recognized for many years (Chen, 1980). The results with this typical D-xylose isomerase are consistent with Co^{2+} being superior to Mg^{2+} as protector against thermal denaturation (Callens *et al.*, 1986).

Although many isomerases have been characterized, information about direct metal ion interactions is very restricted. As reported by Danno (1971), Co^{2+} is bound to D-xylose isomerase from *Bacillus coagulans* in a molar ratio of 1 equiv./monomer. Schray & Mildvan (1972) published binding data from n.m.r. studies. The data fitted to an equation that assumed independent binding of Mn^{2+} at approximately three tight-binding sites/molecule of protein with $K = 3.7 \times 10^4$ M^{-1} and approximately 21 weaker metal-ion-binding sites/molecule of protein with $K = 8.5 \times 10^2$ M^{-1} for D-xylose isomerase from *Streptomyces* sp. A similar study by the same authors with D-xylose isomerase from *Lactobacillus brevis* indicated 2.1 tight Mn^{2+}-binding sites ($K = 1.0 \times 10^5$–1.6×10^5 M^{-1}) and 4.4 weaker Mn^{2+}-binding sites ($K = 5.0 \times 10^4$ M^{-1}). D-Xylose isomerase from *Streptomyces griseofuscus* was found to contain four Co^{2+} ions/molecule of enzyme. One of the four Co^{2+} ions was very tightly bound to the enzyme and had an essential role in maintaining the ordered conformation, especially the quaternary structure, of the enzyme (Kasumi *et al.*, 1981, 1982). Danno (1971), Young *et al.* (1975) and Kasumi *et al.* (1982) all postulated that the role of the bivalent cations in the active ternary metal ion–substrate complexes appears to be structural rather than being directly involved in the catalytic process.

In the present paper we report our direct binding studies of Co^{2+}, Mg^{2+} and Ca^{2+} with the use of equilibrium dialysis and difference absorbance spectrometry. To deduce the structure of the metal ion environment, Co^{2+}-substituted isomerase is preferred, since Co^{2+} ions appear to be more suitable as a spectrometric probe.

EXPERIMENTAL

Materials

The cation salts $CoCl_2,6H_2O$, $MgCl_2,6H_2O$ and $CaCl_2,2H_2O$ were pro analysi products from E. Merck, Darmstadt, Germany. D-Sorbitol dehydrogenase from sheep liver and NADH (grade I) were obtained from Boehringer, Mannheim, Germany. Chelex 100 is an analytical-grade chelating resin from Bio-Rad Laboratories, Richmond, CA, U.S.A., and Nitroso R salt was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of analytical grade.

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Table 1. Cobalt measurements with Nitroso R salt: incubation of enzyme with different Co^{2+} concentrations

D-Xylose isomerase (200 μM active sites) was incubated with various Co^{2+} concentrations in 0.01 M-triethanolamine buffer, pH 8.0, at 35 °C for 4 h.

Added Co^{2+} (μM)	Free Co^{2+} measurement (μM)		
	Enzyme absent	Enzyme present	Co^{2+} deficiency (equiv./monomer)
60	61	8	0.27
80	80	6	0.37
100	99	11	0.44
120	119	14	0.53
160	160	18	0.71
200	202	31	0.86
250	248	64	0.92
300	301	99	1.01
400	400	204	0.98

Enzyme preparation and assay

D-Xylose isomerase from *S. violaceoruber* (L.M.G. 7183) was purified to a homogeneous state as described previously (Callens *et al.*, 1985).

The enzyme concentrations were routinely estimated from the u.v. absorption at 280 nm by using $A^{1\%}_{1\,cm} = 10$ and a tetrameric M_r of 171000 (Callens *et al.*, 1985). The enzyme concentrations were expressed as μM active sites, and the enzyme activity was monitored by the coupled D-xylose isomerase/D-sorbitol dehydrogenase assay as described by Callens *et al.* (1986) and Kersters-Hilderson *et al.* (1987).

Metal-free enzyme was prepared as previously reported (Callens *et al.*, 1986). After EDTA treatment, the enzyme was analysed for residual metal contamination by atomic-absorption spectrometry. Only 0.03 equiv. of Co^{2+}, 0.016 equiv. of Mn^{2+}, 0.14 equiv. of Mg^{2+} and 0.05 equiv. of Ca^{2+}/monomer remained. Furthermore, the activity was less than 3% of that observed in the presence of 10 mM-Mg^{2+}. All buffers were treated with Chelex 100 and stored in acid-washed plastic containers.

Metal analysis

Metal concentrations were determined by atomic-absorption spectrometry. A Perkin–Elmer model 503 atomic-absorption/flame spectrometer was used for Mg. Ca was analysed with a graphite-furnace Hitachi model 180–70 Zeeman atomic-absorption spectrometer, and Co and Mn were analysed with a graphite-furnace Perkin–Elmer model 3030 atomic-absorption spectrometer with $^2H_2$ background correction.

Concentrations of Co^{2+} ions were also determined by a modification of the Nitroso R salt method with disodium 1-nitroso-2-hydroxynaphthalene-3, 6-disulphonate (Vogel, 1962). The standard assay mixture contained 0.7 ml of 1.73 M-sodium acetate and 0.2 ml of 2% (w/v) Nitroso R salt in double-distilled water. After addition of the Co^{2+} sample (100 μl) and mixing, the colour intensity was read immediately at 510 nm in a thermostatically controlled (25 °C) Vitatron photometer, and the Co^{2+} concentrations were calculated by using a calibration factor of 1490 ± 14 M^{-1}.

Equilibrium binding studies

Equilibrium dialysis was carried out in Perspex half-cells (Myer & Schellmann, 1962) separated by a semi-permeable membrane [16 mm ($\frac{5}{8}$ in) dialysis tubing from A. H. Thomas, Philadelphia, PA, U.S.A.]. Before use the half-cells and tubing were treated with 10 mM-EDTA and rinsed extensively with double-distilled water. Equal volumes (200 μl) of enzyme and Co^{2+} ligands, dissolved in 0.15 M-NaCl/0.01 M-triethanolamine/HCl buffer, pH 8.0, were loaded on both sides of the membrane. The cells, sandwiched in a holder, were allowed to equilibrate under rotation (100 rev./min) in a thermostatically controlled (35 °C) Gallenkamp orbital incubator. After an equilibration time of 4 h at 35 °C, the portions were withdrawn from each half-cell and Co^{2+} concentrations were determined as described above. The extent of Co^{2+} binding was calculated from the difference in Co^{2+} concentration in protein-containing and protein-free compartments. The data were analysed by Scatchard (1949) plots.

Difference spectrophotometry

U.v. difference and visible absorption spectra were obtained with a Uvikon-810 double-beam spectrophotometer with two thermostatically controlled (25 °C) 2 cm × 0.437 cm mixing cuvettes (Yankeelov, 1963). Equal volumes (800 μl) of enzyme and metal ion solution in 0.01 M-triethanolamine/HCl buffer, pH 8.0, were added to the compartments of the reference and sample cuvette. After base-line recording, the sample cuvette was mixed and the spectrum was scanned.

For difference absorption titrations, two ordinary 1 cm × 1 cm × 4 cm cuvettes were used. The titrations were performed as described by De Boeck *et al.* (1982). For u.v. difference absorption titrations both cuvettes were filled with 2022 μl of enzyme solution. After base-line recording, portions of the metal ion solution were added to the sample cuvette, whereas an equivalent volume of buffer was added to the reference cuvette. For visible-absorption titrations the sample cuvette was filled with 2022 μl of protein solution and the reference cuvette with an equal volume of buffer. After base-line recording, both cuvettes were titrated with the same metal ion solution.

RESULTS AND DISCUSSION

Evidence for Co^{2+} binding to a high-affinity site

Metal-free enzyme was incubated for 4 h with various amounts of Co^{2+} (Table 1) or for different periods of time with a constant concentration of Co^{2+} (Table 2). Cobalt concentrations were then determined with Nitroso R salt and compared with a similar set of experiments without enzyme. From the data in Tables 1 and 2 it follows that approximately $n-1$ Co^{2+} ions react with the Nitroso R salt. This suggests a direct and tight binding of 1 equiv. of Co^{2+}/monomer.

Evidence for very slow dissociation of the Co^{2+}–enzyme complex was obtained from spectral changes (510 nm) in the presence of Nitroso R salt. Fig. 1 illustrates that, immediately after mixing the enzyme with 1 equiv. of Co^{2+}/monomer, only small amounts of free Co^{2+} could be determined. Long incubation periods with Nitroso R salt, however, finally resulted in complete reaction of the initially added Co^{2+} with Nitroso R salt.

Table 2. Cobalt measurements with Nitroso R salt: different incubation periods of enzyme with a constant concentration of Co^{2+}

D-Xylose isomerase (82.5 μM active sites) was incubated with 250 μM-Co^{2+} in 0.01 M-triethanolamine buffer, pH 8.0, at 35 °C for different periods of time.

Time	Free Co^{2+} determined (μM)	Co^{2+} deficiency (equiv./monomer)
0 min	177	0.86
1 min	176	0.90
10 min	174	0.92
20 min	172	0.95
30 min	170	0.97
60 min	169	0.98
2 h	165	1.03
6 h	166	1.02
25 h	160	1.09

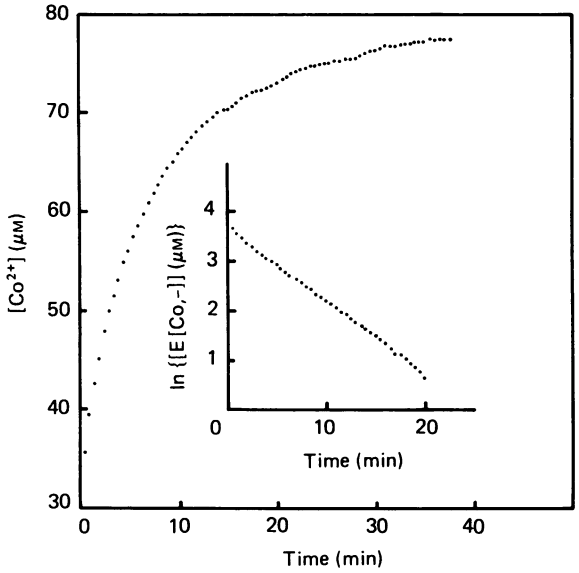

Fig. 1. Dissociation of Co^{2+}-enzyme complex in the presence of Nitroso R salt

D-Xylose isomerase (77.7 μM active sites) was incubated with 75.0 μM-Co^{2+} in 0.01 M-triethanolamine buffer, pH 8.0, at 35 °C.

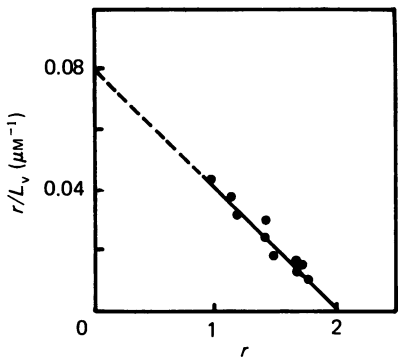

Fig. 2. Equilibrium binding of Co^{2+} to D-xylose isomerase from *S. violaceoruber*

The data from equilibrium-dialysis experiments at 35 °C are plotted in accordance with the Scatchard equation. Equal volumes (200 μl) of enzyme (42 μM active sites) and 20–408 μM-Co^{2+} in 0.15 M-NaCl/0.01 M-triethanolamine buffer, pH 8.0, were loaded on both sides of the membrane. The amount of bound Co^{2+} was estimated from the difference in Co^{2+} between the two half-cells.

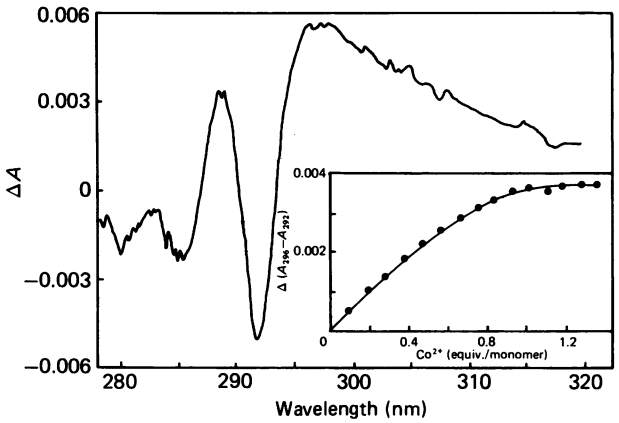

Fig. 3. U.v. absorption spectrum of D-xylose isomerase obtained in the presence of Co^{2+} ions

Conditions were as follows: enzyme (18.5 μM active sites) and 0.5 mM-Co^{2+} in 0.01 M-triethanolamine buffer, pH 8.0, at 29 °C. The inset shows the absorbance difference $\Delta(A_{296}-A_{292})$ of D-xylose isomerase (16.9 μM) versus equiv. of Co^{2+}/monomer at 35 °C.

Linearization of this slow Co^{2+}–nitroso complex-formation yielded a dissociation rate constant of 0.148 min^{-1}.

Total Co^{2+} could also be determined after drastic treatment of the enzyme with SDS at 100 °C, concomitant with dissociation into subunits (results not shown).

Equilibrium binding of Co^{2+}

Further quantitative measurements of the interaction of Co^{2+} with D-xylose isomerase were made by equilibrium dialysis. The data, plotted in accordance with the Scatchard equation (Fig. 2), indicate two binding sites/monomer. Information concerning the first Co^{2+} site could not be obtained since systematic deviations (results not shown) occurred for $n < 1$. Binding of Co^{2+} to this site is probably too strong, and concentrations of remaining free Co^{2+} are too low for accurate determination by atomic-absorption spectrometry. Only the lower-affinity Co^{2+} site could be adequately characterized as having an association constant of $4.0 \times 10^4 \pm 0.4 \times 10^4$ M^{-1}.

Co^{2+}-induced u.v. difference spectra

D-Xylose isomerase undergoes conformational changes on binding of Co^{2+} ions, as shown by u.v. difference absorption spectrometry (Fig. 3). The difference absorption spectrum between Co^{2+}-free enzyme and Co^{2+}-containing enzyme exhibits two absorption maxima at 296 and 289 nm and one absorption minimum at 292 nm, indicating a conformational change affecting the environment of tryptophan.

The difference absorbance change between the maximum at 296 nm and the minimum at 292 nm is a reliable parameter for monitoring the binding of Co^{2+} (Fig. 3

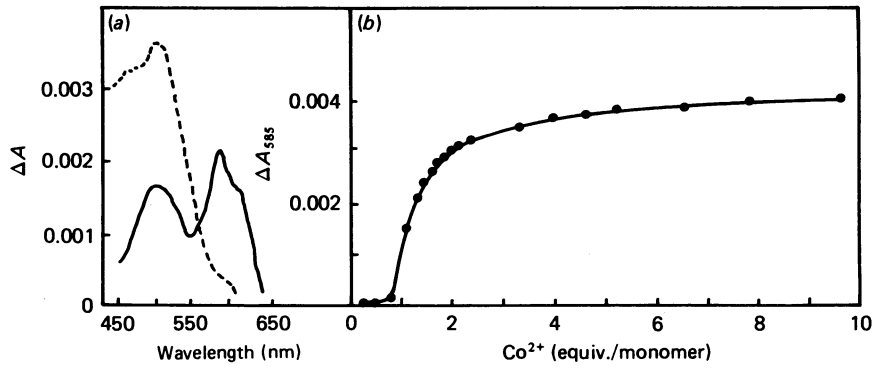

Fig. 4. (*a*) Visible absorption spectrum of Co^{2+}-containing D-xylose isomerase and (*b*) plot of absorbance change at 585 nm versus equiv. of Co^{2+}/monomer

Conditions were as follows: (*a*) enzyme (18.5 μM active sites) and 0.5 mM-Co^{2+} in 0.01 M-triethanolamine buffer, pH 8.0, at 29 °C; the broken line is the spectrum of 0.5 mM-Co^{2+} in 0.01 M-triethanolamine buffer, pH 8.0; (*b*) enzyme (24 μM active sites) titration with Co^{2+}, in 0.01 M-triethanolamine buffer, pH 8.0, at 35 °C, as described in the Experimental section.

inset). After the addition of 1 equiv. of Co^{2+}/monomer the change in absorbance remains constant at its maximum. Thus it is due to filling of only one site/monomer. Assuming a bimolecular association, $E + Co^{2+} \rightleftharpoons E[Co,-]$ (E[Co,−] and E[Co,Co] represent enzyme–metal-ion complexes with Co^{2+} on first site and Co^{2+} on both sites respectively), a binding constant of $\geqslant 3.3 \times 10^6$ M^{-1} can be calculated.

Visible absorption spectra of Co^{2+}–enzyme complexes

Addition of excess Co^{2+} to apoenzyme induces a visible difference absorption spectrum of the Co^{2+}-containing enzyme. The difference absorption spectrum between the Co^{2+}–enzyme complex and free Co^{2+} ions in 0.01 M-triethanolamine buffer, pH 8.0, is shown in Fig. 4(*a*). The spectrum of free Co^{2+} (broken line), exhibiting a structured absorption around 500 nm with low intensity ($\epsilon < 6.9$ $M^{-1} \cdot cm^{-1}$), is characteristic for octahedral symmetry (Cotton & Wilkinson, 1980). In contrast, the difference spectrum of Co^{2+}-containing enzyme exhibits maxima at 585 nm (ϵ 135 $M^{-1} \cdot cm^{-1}$) and 500 nm (ϵ 104 $M^{-1} \cdot cm^{-1}$) and a minimum at 550 nm. These spectral features are not compatible with octahedral symmetry, but are rather compatible with penta- or tetra-co-ordinate Co^{2+} complexes. However, the exact co-ordination geometry remains tentative, since tetrahedral or penta-co-ordinate Co^{2+} complexes are not easily distinguished by visible absorption spectra. Differentiation can only be made on the basis of subtle intensity considerations, tetrahedral chromophores having a larger intensity of electronic absorption than do penta-co-ordinate complexes (Bertini & Luchinat, 1984).

A plot of change in A_{585} versus equiv. of Co^{2+}/monomer is shown in Fig. 4(*b*). Up to the addition of 1 equiv. of Co^{2+}/monomer no spectral changes were observed, in contrast with the u.v. difference spectra, where changes were only observed up to the addition of 1 equiv. of Co^{2+}/monomer. This points to two non-identical Co^{2+} sites, as previously demonstrated by equilibrium-dialysis experiments. Since there is no absorbance change between mono-Co^{2+}–enzyme and solvated octahedral Co^{2+}, the same co-ordination geometry has to be ascribed to the Co^{2+} liganded on the first site. From Fig. 4(*b*) it follows that addition of more than 1 (and up to 10) equiv. of Co^{2+}/monomer causes an increase in the A_{585}. From the titration curve an apparent association constant near 10^5–10^4 M^{-1} could be estimated, in agreement with the value obtained from equilibrium dialysis for the second, lower-affinity, Co^{2+} site.

As a general conclusion the spectrometric studies of Co^{2+}-containing enzyme clearly demonstrate binding of Co^{2+} to two environmentally different sites. The first bimolecular association, $E + Co^{2+} \rightleftharpoons E[Co,-]$, produces changes in the u.v. spectrum of the enzyme, and yields an association constant $K \geqslant 3.3 \times 10^6$ M^{-1}. Co^{2+} bound at this conformational site appears to have octahedral symmetry. The second bimolecular association, $E[Co,-] + Co^{2+} \rightleftharpoons E[Co,Co]$, produces changes in the visible Co^{2+} spectra, and yields an association constant 10^5–10^4 M^{-1}. Co^{2+} binding to this second, lower-affinity, site is involved in the catalytic process, as shown by kinetic studies (M. Callens, unpublished work), and appears to be tetra- or penta-co-ordinate, as deduced from spectral intensities.

Competitive interactions of Co^{2+}, Mg^{2+} and Ca^{2+}

In the following experiments the interactions between Mg^{2+}, the most activating ion, Co^{2+}, the most stabilizing ion, and Ca^{2+}, an ineffective ion, were studied (Callens *et al.*, 1986).

Spectrometric studies of apoenzyme with Mg^{2+} and Ca^{2+} also resulted in a modified u.v. absorption spectrum (Fig. 5). The conformational changes of the enzyme, affecting the tryptophan and tyrosine environment, are very similar to what has been observed with Co^{2+}. The difference absorption spectrum of Mg^{2+}-containing enzyme exhibits two maxima at 296 and 289 nm and one minimum at 292 nm. The change in absorbance between 296 and 292 nm was virtually complete after the addition of 3 equiv. of Mg^{2+}/monomer and a binding constant of 1×10^5 M^{-1} was calculated (results not shown).

Addition of Ca^{2+} to the apoenzyme resulted in a difference absorption spectrum with maxima at 296 and 287 nm and a minimum at 291 nm (Fig. 5). The plot of spectral increases between 296 and 291 nm versus Ca^{2+} increases up to the addition of 3 equiv. of Ca^{2+}/monomer and yielded an association constant $K = 5 \times 10^5$ M^{-1} (results not shown).

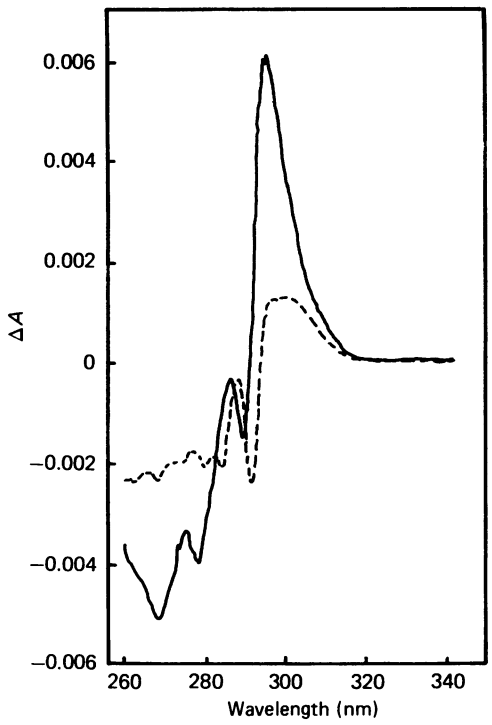

Fig. 5. U.v. absorption spectra of D-xylose isomerase obtained in the presence of Mg^{2+} and of Ca^{2+}

Conditions were as follows: enzyme (15 μM active sites) and 152 μM-Mg^{2+} (----) or 78.1 μM-Ca^{2+} (——) in 0.01 M-triethanolamine buffer, pH 8.0, at 35 °C.

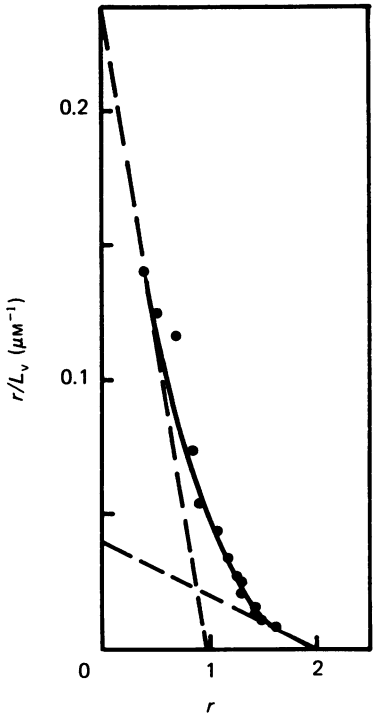

Fig. 6. Equilibrium binding of Co^{2+} to D-xylose isomerase in the presence of 10 mM-Mg^{2+}

The data from equilibrium-dialysis experiments at 35 °C are plotted in accordance with the Scatchard equation. Equal volumes (200 μl) of enzyme solution (34 μM active sites) and 15–500 μM-Co^{2+} in the presence of 10 mM-Mg^{2+} in 0.01 M-triethanolamine buffer, pH 8.0, were loaded on both sides of the membrane. The amount of bound Co^{2+} was estimated from the difference in Co^{2+} between the two half-cells.

The almost identical positions and shapes of the difference spectra between metal-free enzyme and mono-Co^{2+}–, mono-Mg^{2+}– and mono-Ca^{2+}–protein states seem to suggest binding at the same conformational site, or at least co-ordination with some identical ligands.

Interaction of metal-free enzyme with Co^{2+} in the presence of Mg^{2+} was studied by equilibrium dialysis (Fig. 6). The data are consistent with a biphasic Scatchard plot, representative for two different and independent Co^{2+} sites. In contrast with Fig. 2 (absence of Mg^{2+}), no systematic deviations occurred for $n < 1$, which allowed calculation of both $K_{app.}$ values. The slopes yielded $K_{app.1} = 2.5 \times 10^5$ M^{-1} and $K_{app.2} = 2 \times 10^4$ M^{-1}. These findings, and the fact that both $K_{app.}$ values are smaller than the association constants for the two Co^{2+} sites, indicated competition between Co^{2+} and Mg^{2+} for the same binding sites.

Competition between Co^{2+}, Mg^{2+} and Ca^{2+} for the second, lower-affinity, site has been confirmed by kinetic experiments (M. Callens, unpublished work) and by spectroscopic displacement of the Co^{2+} visible absorption spectrum. Fig. 7 shows the effect of the gradual addition of Ca^{2+} to Co^{2+}-loaded enzyme, which resulted in total displacement of Co^{2+}. Gradual addition of Mg^{2+} gave only partial substitution, owing to low association of Mg^{2+} with this site (results not shown).

In general, the competition experiments monitored by means of equilibrium dialysis and spectrometric displacement studies provide evidence for metal ion binding at the same, if not at very close, binding sites.

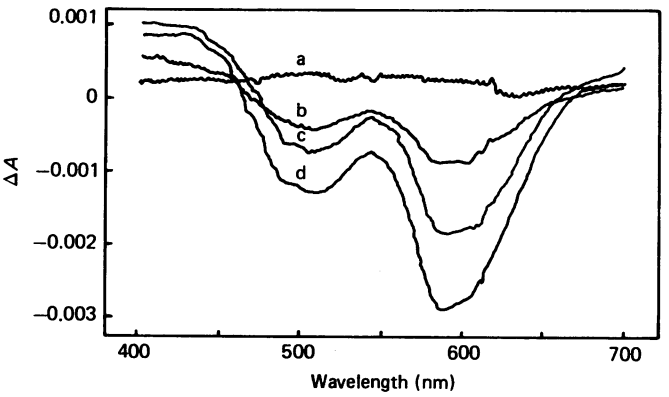

Fig. 7. Visible difference spectrum of Co^{2+}-loaded D-xylose isomerase in 0.01 M-triethanolamine buffer, pH 8.0, at 35 °C

Conditions were as follows: enzyme (23 μM active sites) and 237 μM-Co^{2+} in the sample cuvette, and 237 μM-Co^{2+} in buffer in the reference cuvette. Spectrum a, base-line recording with visible Co^{2+} difference absorption spectrum in memory; spectrum b, displacement of Co^{2+} spectrum after addition of 73 μM-Ca^{2+}; spectrum c, displacement of Co^{2+} spectrum after addition of 237 μM-Ca^{2+}; spectrum d, displacement of Co^{2+} spectrum after addition of 658 μM-Ca^{2+}.

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