ALP Inhibitors: Vanadyl(IV) Complexes of Ferulic and Cinnamic Acid

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Z. Naturforsch. **60b,** 305 – 311 (2005); received September 13, 2004

Two new vanadyl(IV) carboxylate complexes have been obtained: $Na_2[VO(Fer)_2(CH_3OH)_2]$ and $Na_2[VO(Cin)_2(CH_3O)_2]$ and characterized by elemental analysis and UV-vis, diffuse reflectance and IR and Raman spectroscopies ($FerH_2$ = ferulic acid, CinH= cinnamic acid). The thermal behavior was also investigated. The inhibitory effect on alkaline phosphatase activity was tested for the compounds and ferulic and cinnamic acids as well as for the vanadyl(IV) complex of quinic acid for comparison. The ferulic complex together with the free ligands exhibited the lowest inhibitory effect, while the VO/quinic and VO/cinnamic complexes showed an intermediate inhibition potential.

Key words: Vanadium, Cinnamic Complexes, Ferulic Complexes, Alkaline Phosphatase Inhibitors

Introduction

Vanadium, as an early transition metal, exhibits an extraordinarily rich chemistry. It can be readily converted between oxidation states under mild conditions and then spans an unsurpassed range of reactions under environmental conditions including bioaccumulation by organisms. This process probably involves interactions with biological ligands by forming strong complexes with carboxylate, aryloxide and alkoxide functionalities [1 – 3]. Its biological and pharmacological activity is an area of increasing research and widespread interest. Indeed, vanadium compounds are renowned for their potent insulin action both *in vitro* and *in vivo* [4, 5] Several vanadium complexes with insulin-like properties in glucose regulation and metabolism including carboxylate-derived ligands have been reported [6, 7]. Even though only one variable alone cannot explain the enhancement of insulin effects exerted by vanadium compounds, the main action seems to be the inhibition of enzymes catalyzing phosphate ester displacement reactions in the insulin signaling pathway [6 – 8]. The inhibitory effect of vanadyl(IV) complexes upon alkaline phosphatases (ALP) has recently been examined [9 – 12].

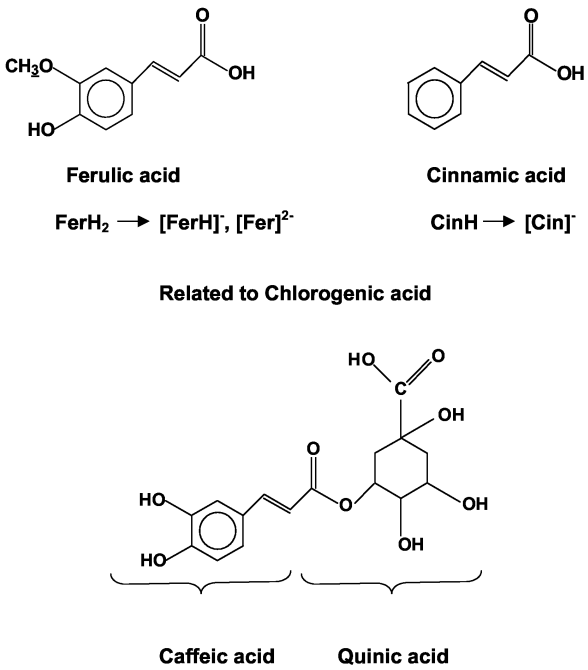

Fig. 1. Schematic structure of cinnamic, ferulic and the related chlorogenic acid.

Ferulic and Cinnamic acids (Fig. 1) are present in Nature and have been selected as natural biological ligands for the vanadyl(IV) cation. Ferulic acid ($FerH_2$) has been found in products of the alkaline oxidation of lignosulfonates, alkaline extracts of hardwoods and a variety of grains [13]. Also this compound is structurally related to cinnamic (CinH) and caffeic acids (the latter is a chlorogenic acid component together

with quinic acid) [1, 2]. Hydroxycinnamic acids are rich in phenolic antioxidants and may reduce the incidence of degenerative diseases, such as cardiovascular disease and cancer, whose mechanism of action is believed to be initiated by free radicals.

The extracts prepared from the leaves of *Cecropia obtusifolia* (whose main constituents are caffeoylquinic and chlorogenic acids) have been traditionally used for the treatment of diabetes. Their effect is to lower plasma glucose concentrations in streptozotocin-induced diabetic rats [14]. Furthermore, chlorogenic acid is a specific inhibitor of the glucose-6-phosphate translocase, a component of the enzyme glucose-6-phosphatase [15]. On the other hand, the products of its hydrolysis, quinic and caffeic acids, are shown to be inactive [16].

The aim of this work is to determine the potential insulin mimetic activities of ferulic and cinnamic acids and their new vanadyl(IV) complexes by a study of their *in vitro* inhibition of phosphatase alkaline enzyme.

Experimental Section

Materials

Reagents were of analytical grade and were used without further purification. Cinnamic and ferulic acid were purchased from Sigma. Methanol (Merck) and diethyl ether (Merck) were dried over 4 Å molecular sieves. $VO(acac)_2$ [17] and $Na_4[VO(Quin)_2SO_4].4H_2O$ [18] were prepared and purified according to literature procedures.

Instrumentation

IR spectra of powdered samples were measured with a Bruker IFS 66 FTIR-spectrophotometer from 4000 to 400 cm^{-1} in the form of pressed KBr pellets. Dry box was used in the case of thermally decomposed samples. Raman spectra were obtained with a Spex-Ramalog double monochromator spectrometer, using the 514.5 nm line of an argon ion laser for excitation. The rotating disk technique was used, in order to avoid burning of the compound by the laser light. Electronic absorption spectra were recorded on a Hewlett-Packard 8453 diode-array spectrophotometer, using 1 cm quartz cells. Diffuse reflectance spectra were obtained with a Shimadzu UV-300 instrument, using MgO as an internal standard. Thermogravimetric (TG) and differential thermal analysis (DTA) were performed on a Shimadzu system (models TG-50 and DTA-50 respectively), working in an oxygen flow (60 ml/min) and at a heating rate of 10 °C/min. Sample quantities ranged between 10 and 20 mg. Al_2O_3 was used as a DTA standard. Vanadium and sodium contents were determined by the tungsto-phosphovanadic method [19] and by flame photometry, respectively. C and H were determined using a Carlo Erba EA 1108 equipment.

Preparative

Synthesis of the vanadium compounds was performed under a high-purity nitrogen atmosphere using standard Schlenk techniques. Solvents were dried and saturated with dry nitrogen. $VO(acac)_2$ was freshly prepared prior to its use for the synthesis of the complexes [20].

$Na_2[VO(Fer)_2(CH_3OH)_2]$, (Fer = Fer^{2-}): Vanadyl acetylacetonate (1 mmol, 0.265 g) was disssolved in methanol (10 ml) with magnetic stirring at 50 °C for 1 h. The hot solution was filtered and solid ferulic acid (2 mmoles, 0.388 g) was added in small portions under continuous stirring. The pH was adjusted at 7.0 using freshly prepared sodium methoxide. The reaction mixture was allowed to reflux at 50 °C for 2 h and then evaporated to dryness using a rotatory evaporator. The green powder obtained was washed several times with warm ethyl acetate in order to eliminate the excess of the ligand. The purity of the solid was tested by IR spectroscopy selecting the band at *ca.* 1692 cm^{-1} which corresponds to the free carboxylic acid group. The solid was finally dried in an oven at 60 °C. Yield: 65%. $C_{22}H_{24}O_{11}VNa_2$ (561): calcd. C 47.1, H 4.3, Na 8.2, V 9.1; found C 47.5, H 4.1, Na 7.8, V 8.8. TG/DTA: weight loss of 11.4% was observed below 120 °C; calcd. 11.6 for $2CH_3OH$. Endothermic peak was observed at 107 °C. Exothermic signals were observed at 210, 274, 441 and 673 °C.

Ferulic sodium salt: An aqueous solution of sodium hydroxide (1 mmol in 10 ml) was added to a solution of ferulic acid (1 mmol, 0.388 g) in methanol (20 ml) with gentle stirring. Addition of excess acetone produced a yellow precipitate, which was filtered, washed with acetone and dried in air. Yield: 82%.

$Na_2[VO(Cin)_2(CH_3O)_2]$, (Cin = Cin^-): A similar procedure was followed to prepare the complex (70% yield). Vanadyl acetylacetonate and cinnamic acid were used. The compound was washed several times with diethyl ether. $C_{20}H_{20}O_7VNa_2$ (469): calcd. C 51.2, H 4.7, Na 9.8, V 10.9; found C 51.0, H 4.5, Na 10.3, V 11.3. TG/DTA: no weight loss was observed below 190 °C. Decomposition gradually began with exothermic peaks at 195, 243, 327 and 398 °C.

Cinnamic sodium salt: 10 ml of an aqueous solution of potassium hydroxide (1 mmol, 0.056 g) was added to a solution containing cinnamic acid (1 mmol, 0.148 g) in 10 ml of water. After stirring, the resulting solution (pH = 12) was left to evaporate until a white precipitate occured. The solid was isolated by filtration, washed twice with ethanol and dried in an oven at 60 °C. Yield: 85%.

Spectrophotometric titrations

Compositional studies were carried out using spectrophotometric titrations. Each ligand was dissolved in methanol

(final concentration: 0.01 M) and $VO(acac)_2$ was added in ligand-to-metal ratios from 10 to 0.5, in an inert atmosphere and adjusting the final pH value to 7.0 using sodium methoxide.

Alkaline phosphatase assays

The effect of ferulic and cinnamic acids, vanadyl(IV) cation and the different VO/complexes on ALP activity was determined spectrophotometrically. The reaction was started by the addition of the substrate (p-NPP), and the generation of p-nitrophenol was monitored by the absorbance changes at 405 nm. Briefly, the experimental conditions for ALP specific activity measurement were as follows: 1 μg/ml of bovine intestinal ALP and 5 mM of p-NPP were dissolved in the incubation buffer (55 mM glycine + 0.55 mM $MgCl_2$, pH = 10.5) and held for 10 minutes. The effects of the compounds were determined by addition of different concentrations (1 – 100 μM) of each compound to the pre-incubated mixture. The effect of each concentration was tested at least in triplicate in three different experiments. The initial rate, in absence of any compound (V_0), was determined as the rate of p-NPP hydrolysis at 37 °C and pH = 10.5. The enzymatic reaction rates inhibited by vanadium compounds, V_i, were determined like V_0 but in the presence of the different concentrations of each of the investigated systems. Data were expressed as mean±SEM (SEM = standard error of the mean). Statistical differences were analyzed by Student's *t*-test.

Results and Discussion

UV-vis and diffuse reflectance spectra

The electronic spectrum of the system VO/Fer has been measured using a methanolic 1/2 solution because of the low solubility of the solid complex in many solvents. The electronic absorption bands of the UV-vis spectra of solutions of VO/Cin and $VO(acac)_2$ in methanol are also shown in Table 1.

According to the well-known Ballhausen and Gray scheme [21] the d-d absorption spectra of VO(IV) complexes with square pyramidal geometry (C_{4v} symmetry) present three bands corresponding to the transitions $b_2 \rightarrow e$, $b_2 \rightarrow b_1$, and $b_2 \rightarrow a_1$. The latter is usually masked by charge-transfer bands. The splitting of the absorption bands is indicative of the distortion of the square pyramidal geometry [22]. The presence of the same pattern in the absorption spectra is distinctive of the same binding modes of the ligands. A C_{4v} geometry can be inferred for the three complexes shown in Table 1. Although the coordination sphere of the VO/cinnamic complex is suggested to be composed of $2\times(COO^-$, R-$O^-)$, the splitting of the ^2E (d_{xy}, d_{yz}) term into two levels such as for α-hydroxycarboxylic acids [22] is not observed. In the present cases the symmetry of the complexes is not lowered to C_{2v}. The blue shifts of the absorption bands of the two complexes compared with the spectra of $VO(acac)_2$ are indicative of a weaker crystal field produced by the negative charge of the interacting carboxylate groups of ferulic and cinnamic acid together with methanol or methoxide groups in the coordination sphere, respectively [23].

Table 1. Electronic absorption bands: UV-vis for $VO(acac)_2$, $VO(acac)_2$: Ferulic acid (1 : 2) in methanol (pH = 7.0) and $Na_2[VO(Cin)_2(CH_3O)_2]$ in methanol, and diffuse reflectance of the solid complexes.

	UV-vis[a]		Diffuse reflectance[a]
	$b_2 \rightarrow b_1$	$b_2 \rightarrow e$	
$VO(acac)_2$	580 (12)	778 (36)	590, 705
VO / Fer	582 (32)	790 (73)	630, 840
VO / Cin	570 (48)	816 (68)	600, 706, 830

[a] Wavelength, nm. Molar extintion coefficient, $M^{-1}cm^{-1}$ in parentheses.

In the solid state of $VO(acac)_2$ the V atom is five-coordinated [6]. This complex forms adducts upon dissolution in organic solvents, generating octahedral complexes $[VO(acac)_2L]$. The coordination of the solvent methanol (Table 1) produces a shift in the $b_2 \rightarrow e$ absorption bands of $VO(acac)_2$ to lower energies. In Table 1 the diffuse reflectance spectra of the two complexes are compared with the spectrum of $VO(acac)_2$. The $b_2 \rightarrow b_1$ and $b_2 \rightarrow e$ electronic bands are blue shifted in the solid state for VO/Fer and VO/Cin complexes. This behavior is typical of vanadyl(IV) complexes coordinated to carboxylate groups.

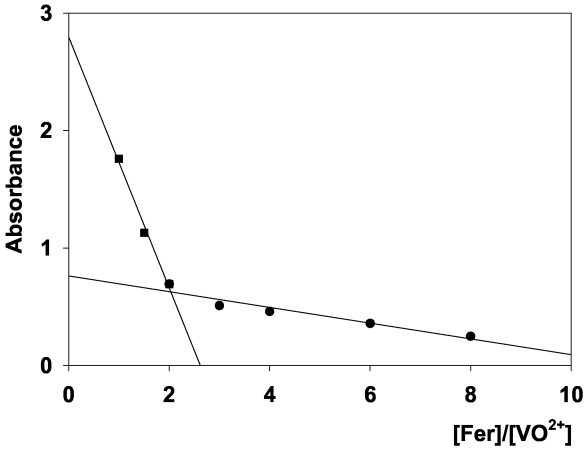

Fig. 2. Spectrophotometric titration of VO^{2+} with ferulic acid at pH 7.0 for $\lambda = 790$ nm.

I		II	III		Assignments
IR	Raman	IR	IR	Raman	
1692 vs					ν(C=O), carboxylic acid
1624 vs	1629 s	1636 m	1636 m	1636 m	ν(C=C)
		1541 s	1592 s	1592 w	$\nu_{as}(COO^-)$
1517 m	1517 mw	1497 m	1512 s	1487 w	ν_{ring}
		1392 s	1388 s	1393 s	$\nu_s(COO^-)$
1276 m	1271 s	1266 s	1273 m	1279 s	ν(aryl-O)
1035 s		1021 m	1030 m		ν(O-CH_3) (Ferulic/CH_3OH)
			978 s	969 s	ν(V=O)

Table 2. Assignments of some characteristic IR and Raman bands (cm^{-1}) of ferulic acid(I), ferulic sodium salt(II), and $Na_2[VO(Fer)_2(CH_3OH)_2]$(III).

vs: very strong, s: strong, m: medium, w: weak.

I		II	III		Assignments
IR	Raman	IR	IR	Raman	
1684 vs					ν(C=O), carboxylic acid
1629 s	1636 m	1643 m	1636 m	1639 s	ν(C=C)
		1546 m	1533 m	1533 w	$\nu_{as}(COO^-)$
1495 m	1496 w	1498 w	1500 m	1510 m	ν_{ring}
		1407 s	1369 m	1374 m	$\nu_s(COO^-)$
			1027 s	1027 m	ν(O-CH_3) (methoxide)
			938 m	944 s	ν(V=O)

Table 3. Assignments of some characteristic IR and Raman bands (cm^{-1}) of cinnamic acid(I), cinnamic sodium salt(II), and $Na_2[VO(Cin)_2(CH_3O)_2]$(III).

vs: very strong, s: strong, m: medium, w: weak.

Spectrophotometric titrations

In order to obtain an insight into the stoichiometry of the complexes we have performed spectrophotometric titrations in the systems VO/Fer and VO/Cin, monitoring absorbance changes of the $b_2 \rightarrow e$ band as a function of the ligand-to-metal ratio at constant wavelength and pH 7.0 [24]. Fig. 2 shows the data obtained in the VO/Fer system at $\lambda = 790$ nm. Identical results were obtained with the VO/Cin system. The formation of 2:1 (L:M) complexes allows us to confirm that the vanadyl(IV) cation interacts in methanolic solution with the same stoichiometry as in the solid state.

Infrared and Raman spectra

$Na_2[VO(Fer)_2(CH_3OH)_2]$

In the IR and Raman spectra (Table 2) the following changes are observed: The strong band at 1692 cm^{-1} is assigned to the (C=O) stretching of the –COOH group in the free ligand and disappears when the salt and the complex are formed. This bands splits into two components corresponding to antisymmetric and symmetric stretching modes. In the sodium salt the spectrum shows two new bands at 1541 cm^{-1} (ν_{as} COO^-) and 1392 cm^{-1} (ν_s COO^-). A similar behavior is observed in the spectrum of the complex where the bands appear at 1592 and 1388 cm^{-1}, respectively. The difference $\Delta\nu = \nu_a(COO^-) - \nu_s(COO^-)$, is used as a criterion to establish the coordination of the COO^- group to the metal [25–27]. The Δ value (*ca.* 149 cm^{-1}) observed in the sodium salt (IR spectrum) is in concordance with the ionic form for the carboxylate group. Upon coordination with the metal center, the Δ value (*ca.* 204 cm^{-1}) corresponds to a unidentate coordination of the carboxylate moiety. In the Raman spectrum the lowering in the intensity of the antisymmetric mode is in accord with the behavior expected for these bands [25]. The strong intensity of the symmetric mode in the IR spectrum may be due to the superposition of other modes.

The position of the bands, related to the stretching modes of the methoxide group of Ferulic acid (ν(O-CH_3)) remains unaltered upon complexation (IR and Raman spectra) indicating the lack of interaction with the metal center. The ν (O-CH_3) stretching modes corresponding to the methanol solvate molecules is also expected to appear in this region. In order to provide more experimental data for the assignments of these bands, the spectra of the complex thermal decomposition products have been recorded and mutually compared. The definite decrease of the ν(O-H) vibrations of coordinated methanol observed at 3426 cm^{-1} in the complex (Fig. 3) at 120 °C, confirms the presence of the methanol adduct. Furthermore, the ν(CH_3-O) band of coordinating methanol (1030 cm^{-1}) is overlapped by the ν(CH_3-O-) of the ligand [25, 28, 29]. The gradual vanishing of the mode in this spectral region on heating indicates the loss of methanol at 120 °C and the start of the decomposition of the ligand at 220 °C and 270 °C, respectively. On the other hand, the partial decrease of the 2969 and 2941 cm^{-1} and 1457, 1420, 1385 cm^{-1} band intensities confirms the contribution

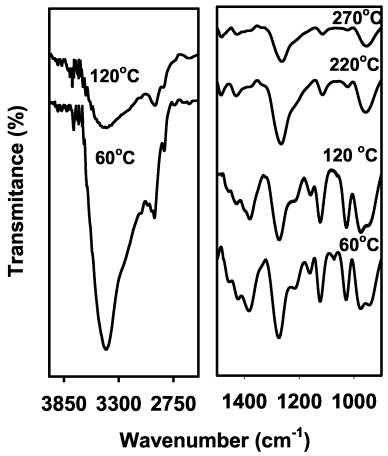

Fig. 3. Selected parts of the infrared spectra of $Na_2[VO(Fer)_2(CH_3OH)_2]$ measured after heating at different temperatures.

of the $\nu_a/\nu_s(CH_3)$ and $\rho(CH_3)$ vibrations, respectively, in these absorptions (Fig. 3).

The value of ν(V=O) observed in the complex is characteristic of oxovanadium(IV) carboxylates [27].

$Na_2[VO(Cin)_2(CH_3O)_2]$

The IR and Raman spectra showed similar features to those of ferulic acid complex (see Table 3).

The difference $\Delta\nu$ values for the potassium salt ($\Delta\nu = 139$ cm^{-1}) and for the complex ($\Delta\nu = 164$ cm^{-1}) suggest an ionic and unidentate coordination, respectively. The presence of the methoxide group in the coordination sphere of the vanadium center is inferred from the appearance of a strong IR band at 1027 cm^{-1} [30] and the absence of the band related to the OH stretching in the 3500 – 3300 cm^{-1} range. The lower ν(V=O) observed in the VO/Cin with respect to the VO/Fer complexes is indicative of the higher σ-donating power of a methoxide ligand (compared with methanol), increasing the electron density in the metal d orbitals and reducing the $p_\pi \rightarrow d_\pi$ donation from oxygen to vanadium [31, 32]. When the coordination of VO(IV) occurs with deprotonated OH groups of sugars or polyols, this effect is stronger and ν(V=O) bands are located at *ca.* 920 cm^{-1} [33 – 36].

Alkaline phosphatase activity

Alkaline phosphatase (E.C.3.1.3.1) is a metalloenzyme that catalyzes the hydrolysis of phosphate monoesters. The most widely characterized ALP is an

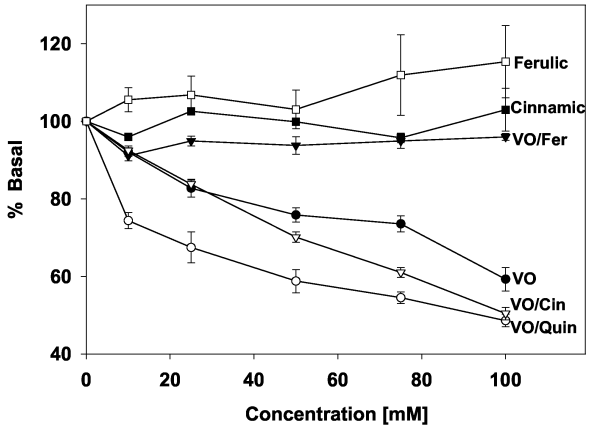

Fig. 4. Effect of cinnamic and ferulic acids, VO/Cinnamic, VO/Ferulic, VO/Quinic and VO(IV) on ALP activity from bovine intestinal mucose. The initial rate was determined by incubation of the enzyme at 37 °C for 10 min in the absence or presence of variable concentrations of the inhibitors. Basal activity was 5.2 ± 0.2 nmol pNP min^{-1} μg^{-1} protein. The values are expressed as mean ±SEM (n = 9).

80,000 molecular weight enzyme from *E. coli*, for which a low-resolution X-ray crystal structure is available [37]. It is a dimer containing three non-equivalent metal-binding sites in each subunit. Two of these are occupied by zinc ions, one with a catalytic role and the other one with a structural function. The third metal is a Mg(II) cation, also playing a structural role.

In Fig. 4 the effects of the oxovanadium(IV) complexes and the free ligands on the ALP activity are shown. In order to compare inhibition effects with other hydroxyacids, the alkaline phosphatase activity of the $Na_4[VO(Quin)_2SO_4].\ 4H_2O$ [18] complex was also tested. The effect of vanadyl(IV) cation is also displayed. Referring to vanadyl(IV) cation as the form of VO^{2+} in the ALP assay medium is misleading since the vanadyl cation is only a small fraction of V(IV) species at neutral or higher pH [8]. Given the pH value used herein (10.5) there is no doubt that the major vanadium(IV) species will be $[VO(OH)_3]^-$. ALP has already been proved to be sensitive to vanadium(IV) and is a good model to test the effect of new vanadium derivatives *in vitro* [8]. Only quinic and VO/Cinnamic complexes are stronger inhibitory agents than vanadium(IV) in the whole concentration range ($p < 0.001$) and at concentrations higher than 50 μM, respectively. On the other hand, the VO/Ferulic complex and the free ligands behaved in a different manner than the others denoting the lack of inhibition on the ALP ac-

tivity. This behavior has been documented for a few vanadium complexes and the lack of inhibitory activity upon phosphatases is explained by compound breakdown in solution. However, the compounds are supposed to be inhibitors if the effect is determined under conditions where they remain intact [38]. It has been demonstrated [36] that the potency of the enzymatic inhibition of the vanadium compounds varies considerably depending on the ancillary ligands. Increasing the bulkiness of the ligands by introducing sustituents significantly decreased the inhibitory effect on the ALP activity. Vanadium compounds having 6-coordinate octahedral geometry display the lower inhibition on ALP activity [38].

The inhibitory effect of vanadium compounds on the activity of the enzymes that catalyze phosphoryl group transference can be attributed to the formation of a trigonal bipyramidal transition state analogue. These phosphorus-mimicking vanadium compounds, with a structure closer to the transition state than that of the phosphorus compounds, likely fit more tightly into the active site, causing inhibition of the enzymatic reaction [37]. Although the trigonal bipyramidal coordination geometry is usual for vanadium(V) but not for the vanadyl(IV) cation, it has been recently demonstrated that this coordination structure can be attained by VO^{2+} in the presence of flexible ligands such as proteins and enzymes [22, 39]. However, in a recent study it has been suggested that the best bioavailability rather than the increased potency at the phosphatase enzyme active sites of vanadyl(IV) complexes compared with inorganic vanadium, would be the cause of their higher insulin mimetic activities [7].

The results obtained with the series of carboxylate/VO complexes suggest:

- that the complexes having a set of hard donor ligands like COO^- and O^- (quinate) or CH_3O^- (from methoxide in the cinnamate complex), that forms particularly strong complexes with the vanadyl cation [40] better adopt the transition state structure of ALP, being in this way stronger inhibitors than the other ones.
- that the effect observed for the VO/Ferulic complex may be attributed to the lability of the coordinated methanol molecules. The complex is thermally unstable (methanol is lost at 103 °C) denoting weak coordination to the vanadyl(IV) cation. This fact suggests a reduction of the possibility to adopt the appropriate transition state symmetry in the interaction with the enzyme in aqueous solution.

Conclusions

Two new vanadyl(IV) complexes with naturally occurring ligands have been prepared and characterized. Their inhibitory effect upon specific alkaline phosphatase activity shows a different behavior probably associated with the lability of the ligands in the coordination sphere. Ferulic and cinnamic acids do not exert inhibition on the enzyme, an effect that was previously determined for quinic and caffeic acids upon glucose-6-phophatase as stated in the introductory section.

Acknowledgements

This work was supported by CONICET, CICPBA, UNLP and ANPCyT (PICT 06-06148). EGF is a member of the Research Career from CONICET. PAMW is a member of the Research Career from CICPBA.

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