Effect of Vanadium Ions on ATP Citrate Lyase

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Abstract. We have shown previously that vanadium ions (vanadate and vanadyl) inhibit autophosphorylation of histidine but not that of serine in ATP citrate lyase (ACL). Here we report the results concerning the effect of monovanadate (+ oligomers), decavanadate as well as vanadyl on the activity of ACL of the rat liver. Susceptibility of ACL to inhibition by vanadate was rather low. Vanadate at concentration 10^{-4} mol/l inhibited ACL by only 10% and at 10^{-3} mol/l concentration monovanadate inhibited ACL by 37%. Decavanadate had comparable potency to inhibit ACL. So was vanadyl which produced 20%, 32% and 66% inhibition at 10^{-4} mol/l, 10^{-3} mol/l and 10^{-2} mol/l concentrations, respectively. From the kinetic data it appears that inhibition by mono- and deca-vanadate of ACL with respect to both ATP and citrate was of competitive nature. Vanadyl inhibited ACL noncompetitively with respect to these substrates. However, all three species of vanadium ions inhibited ACL noncompetitively with respect to CoA. Endogenous (auto)phosphorylation of the ACL histidine as well as its response to vanadate depended on the presence of he substrate (citrate + CoA). The kinetic characteristics of vanadium ions action of ACL was compared with that previously demonstrated for vanadium inhibition of succinyl-CoA synthetase. Plausibility of our hypothesis that inhibition of histidyl phosphorylation at the catalytic site may be a common mechanism by which vanadium ions suppress the activity of the histidyl containing enzymes catalyzing the phosphoryl transfer is discussed.

Key words: ATP citrate lyase – Vanadate – Vanadyl – Histidine phosphorylation

Introduction

Vanadium in its form of pentavalent anion and its polymers as well as tetravalent cation have been shown to exert a great amount of biological effects but their physiological (regulatory) role is still uncertain (for reviews see Erdman et al. 1984;

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Nechay 1984; Nechay et al. 1986a). Vanadium is a trace element essential for growth and normal functioning of living organism but in high doses it may be toxic. The mechanism of either of these effects has not yet been fully elucidated, however.

Vanadium ions, particularly their pentavalent form, vanadate, have been shown to affect also protein phosphorylation, one of the most important control mechanism in the living cell. Swarup et al. (1982) have demonstrated that vanadate inhibits phosphotyrosyl-protein phosphatase activity while that of seryl-protein and tryptophanyl-protein phosphatases remained virtually unaffected. Since then, vanadate stimulation of tyrosyl phosphorylation (mainly by phosphatase(s) inhibition) has been shown to take place in a number of proteins (Gresser et al. 1987). However, stimulation of appropriate kinase(s) has not yet been ruled out (Tamura et al. 1984; Tracey and Gresser 1986). Also other types of protein kinases, for example, cAMP-dependent one, are stimulated by vanadate (Catalan et al. 1982; Křivánek 1984). More recently we have shown that both vanadate and vanadyl can inhibit endogenous phosphorylation of the histidyl residue in the α-subunit of succinyl-CoA synthetase (SCS) of rat brain mitochondria (Křivánek and Nováková 1989). This is the only amino acid residue in this protein which is phosphorylated. Another enzyme containing histidine at its catalytic site and phosphorylation of which is also inhibited by vanadate (Křivánek and Nováková 1991a) is ATP citrate lyase [citrate cleavage enzyme; EC 4.3.1.8 A ATP:citrate oxaloacetate lyase (CoA – acetylating and ATP-dephosphorylating); ACL]. Since histidine phosphorylation of SCS is an essential step in its catalytic mechanism it is not surprising that also activity of SCS is inhibited by vanadate (Křivánek and Nováková 1991b). Vanadate has been used as a tool to distinguish the two forms of SCS (Křivánek and Nováková 1992). ACL contains another phosphorylable amino acid – serine at two chemically distinct, regulatory sites, A and B but their phosphorylation is not affected by vanadate (Křivánek and Nováková 1991a). Otherwise ACL shares several features with SCS (Wells 1991) besides the vanadate sensitive histidyl phosphorylation. Vanadate inhibits also another enzymes containing phosphorylable histidine like glucose-6-phosphatase and 2,3-phosphoglycerate dependent phosphoglycerate mutase (Climent et al. 1981). However, the vanadate effect on their histidine phosphorylation has not yet been tested. The aim of the present experiments is to test the effect of vanadate on ACL activity, to compare it with the vanadate inhibition of SCS and to answer the question whether the mechanism of inhibiton is the same for both enzymes. It is shown that monovanadate, decavanadate as well as vanadyl exert inhibitory effects. Kinetic data revealed similarities between the effect of vanadate on ACL and the previously published effect on SCS. However, in some respects the effects of vanadium ions on ACL are different from those previously showed for SCS. The mechanism of the vanadium ions effect on ACL and SCS does not seem to be identical. Thus our assumption that inhibition of

the phosphoryl transfer enzymes by vanadate may be accounted for by attenuation of their histidine phosphorylation is not unambiguously justified by the present experiments.

Materials and Methods

Chemicals. Tris, dithiothreitol (DTT), coenzyme A were from Sigma Chemical Co. (St. Louis, MO), ATP, disodium salt, vanadate free, was from Boehringer (Mannheim, FRG), vanadyl sulfate from Janssen Chimica (Beerse, Belgium) and orthovanadate from BDH (London, UK). Other chemicals were of analytical grade from the local sources. To prepare "monovanadate" the stock solution of orthovanadate was boiled for 15 min. After several hours pH was adjusted to 7.4. This treatment destroys decavanadate that can be present in the orthovanadate stock solution which, however, may still contain some lower oligomers, mainly di- and tetra-mers. Decavanadate was prepared by titration of the monovanadate solution to pH 4 with HCl.

Enzyme preparation and purification. The enzyme was prepared from rat liver by a slightly modified procedure published previously (Linn and Srere 1979). The rats (hooded males, Long-Evans strain, 250 g b.w.) were starved for two days and then fed on a high sucrose, low fat diet for 3 days before being killed by decapitation. Procedure is based on the fractionation with polyethylene glycol and ammonium sulfate. Two steps for further purification have been used: DEAE-cellulose followed by Biogel A, 1.5 m chromatography. By this procedure a relatively homogenous (polyacrylamide gel electrophoresis) preparation can be obtained (Křivánek and Nováková 1991a).

Enzyme assay. The hydroxamate method (Hestrin 1949) was used for both measurement of the enzyme activity during preparation and for testing the effect of vanadium on its activity. The incubation medium (final volume 250 μl) consisted of (in mmol/l) Tris.Cl, pH 8.4 (200); $MgCl_2$, (10); DTT, (1); citrate, (20); CoA, (1); ATP (6). After 5 min preincubation with the enzyme (about 5 μg prot.) and vanadium the reaction was started by adding ATP and stopped after 30 min incubation at 37 °C by adding 500 μl of the hydroxylamine reagent. Ten min later the same volume of $FeCl_3$ reagent was added and mixed thoroughly. The blanks contained all medium components except CoA. Color density was measured spectrophotometrically at 530 nm. Activity of the enzyme was expressed in micromoles per min and mg prot.

Endogenous phosphorylation. The enzyme preparation was preincubated 3 min at 30 °C in a medium (final volume 50 μl) consisting of (in mmol/l): Tris.HCl, pH 7.4 (30); $MgCl_2$ (10); EGTA, (0.4); [gamma-^{32}P] ATP, (0.02; 4000 cpm per pmol); about 25 μg enzyme protein. The reaction was started by addition of ATP and after 20 s terminated by adding the same volume of the stop solution (Laemmli 1970) and boiling for 3 min. For SDS polyacrylamide slab gel electrophoresis the Laemmli (1970) system was used. Four per cent and 8% polyacrylamide was used for stacking and separation gels, respectively. Fifty μl of the final suspension was applied onto the gel. The gels were stained and destained (Fairbanks et al. 1971). The ACL band was dissected out of the dried gels and transferred into vials containing a scintillation cocktail based on toluene and the radioactivity was measured by scintillation spectroscopy.

Proteins were measured according to Lowry et al. method (1951) using bovine serum albumin as a standard.

For statistical evaluation of the results Student's t-test was used.

Results

In most experiments the hydroxamate method (Hestrin 1949) was used in spite of its applicability for only a narrow range of enzyme concentrations (Linn and Srere 1979). The linearity of time and enzyme concentration dependence of the enzyme activity was obtained for the range of 0.6 to 6.0 μg protein of the enzyme and 5 to 30 min incubation period (not shown). This was the range in which effects of vanadium ions on ACL were tested. Some experiments have been replicated with the use of the enzymatic method (Linn and Srere 1979). Essentially the same results were obtained, but the correction had to be made for the vanadate effect of NADH (not shown). The term monovanadate is used for the solutions of orthovanadate subjected to boiling to remove decavanadate. (See section Materials and Methods.) Therefore, presence of some oligomers, namely di- and tetra-vanadate cannot be ruled out, particularly at the higher vanadate concentrations.

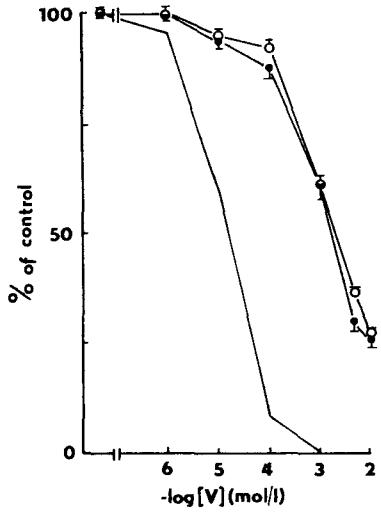

Figure 1. Does dependent inhibition of ATP citrate lyase by monovanadate (•—•) and decavanadate (o—o) as compared to inhibition of succinyl-CoA synthetase (—) by monovanadate (Křivánek and Nováková 1991b). Each point represents the mean of at least four measurements ±S.E.M.

Monovanadate inhibited ACL activity (Fig. 1). Approx. 10% inhibition ($P < 0.05$) was produced by 10^{-4} mol/l concentration, while 37% ($P < 0.01$) and 68% ($P < 0.03$) inhibition took place at 10^{-3} mol/l and 10^{-2} mol/l vanadate, respectively. Decavanadate inhibitory effect was almost identical with that of monovanadate (Fig. 1). Thus ACL appears to be much less sensitive toward vanadate action than SCS for which it has been shown previously that millimolar vanadate exerts almost complete inhibition (Fig. 1). Vanadyl inhibited ACL to the same extent as did vanadate: at 10^{-4} mol/l concentration it produced 20%

inhibition ($P < 0.01$). At 10^{-3} mol/l and 10^{-2} mol/l vanadyl concentrations 32% ($P < 0.01$) and 66% ($P < 0.03$) inhibitions were reached (Fig. 2). This is close to its previously demonstrated effect on SCS (Fig. 2). In contrast to SCS ACL does not reveal differential susceptibility toward either vanadate or vanadyl.

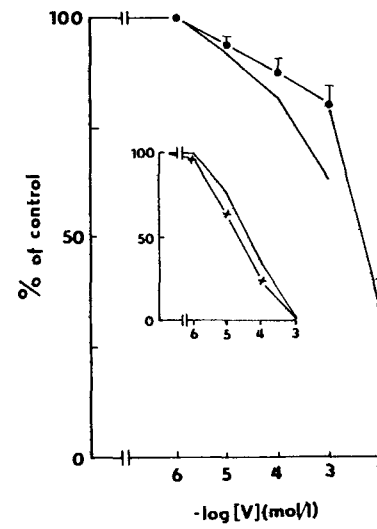

Figure 2. Inhibition of ATP citrate lyase by vanadyl ions (•—•) as compared to that of succinyl-CoA synthetase (—) found previously (Křivánek and Nováková 1991b). Each point represents the mean of at least four measurements ±S.E.M. *Insert.* Inhibition by monovanadate of histidine phosphorylation of succinyl-CoA synthetase (·—·) as well as ATP citrate lyase (×—×) previously published (Křivánek and Nováková 1991a,b).

Vanadate is known to form complexes with various oxocompounds including citrate (Nechay et al. 1986b; Rehder 1991). To see whether effectiveness of vanadate is influenced by substrate, we tested the effect of vanadate on endogenous phosphorylation of ACL in both absence and presence of citrate plus CoA. As shown on Table 1 addition of the substrates considerably depressed both phosphorylation itself and the inhibitory potency of vanadate on the endogenous phosphorylation of ACL (20% and 79% inhibition achieved with and without substrates, respectively.) However, the inhibitory effect of vanadate on the endogenous phosphorylation of SCS was not influenced by substrate (susccinate + CoA). This may at least partially account for the lower efficiency of vanadate to inhibit ACL as compared with SCS.

To estimate the possible mechanism by which vanadium may inhibit ACL, kinetics of the inhibition was studied. The Fig. 3 shows that inhibition by both mono- and deca-vanadate (10^{-3} mol/l) with respect to ATP is of competitive nature, whereas vanadyl (10^{-2}mol/l) inhibited ACL noncompetitively. The inhibition of ACL by monovanadate with respect to citrate was apparently of competitive nature, whereas vanadyl again exerted noncompetitive inhibition (Fig. 4). On the other hand, all three vanadium ion species inhibited ACL noncompetitively with

Table 1. Effect of substrates on the efficiency of vanadate inhibition of endogenous phosphorylation of ATP citrate-lyase (ACL) compared to that of succinyl-CoA synthetase (SCS). Preparations of ACL from rat liver or SCS from rat brain mitochondria were subjected to endogenous phosphorylation with (γ – ^{32}P)ATP and Mg^{2+} in the absence or presence of the respective substrates (citrate + CoA, succinate) both without or with sodium vanadate (10^{-4} mol/l). Results are expressed in mean cpm ± S.E.M. of the bands dissected from the dried polyacrylamide gel electrophoregrams. (For details see Materials and Methods).

Enzyme	Without substrate				With substrate			
	Control	+Vanadate	P	Inhibition (%)	Control	+Vanadate	P	Inhibition (%)
ACL	2536 ± 60 ($n = 4$)	521 ± 30 ($n = 4$)	< 0.003	79	524 ± 28 ($n = 4$)	420 ± 23 ($n = 4$)	< 0.05	20
SCS	280 ± 8 ($n = 3$)	140 ± 25 ($n + 3$)	< 0.01	50	440 ± 29 ($n = 3$)	230 ± 7 ($n = 3$)	< 0.01	48

respect to CoA (Fig. 5). Kinetic study thus suggests a qualitative similarity between the mechanism of vanadate inhibition of ACL as compared with that of SCS. Some differences between the mechanism of inhibition cannot be excluded, however. Lower susceptibility of ACL toward the vanadium and its possible mechanism has been mentioned above. In our previous paper we have shown that vanadate inhibition of SCS activity was not expressed at lower (up to 20 mmol/l) succinate concentrations. It seemed that inhibition was expressed only after some "threshold" concentration of succinate had been reached. (Křivánek and Nováková 1991b). No such dependence could be observed with ACL. A clear-cut inhibition proceeded at the citrate concentration range from 0.5 to 20 mmol/l as can be seen from the kinetic data presented on Fig. 4.

ADP, a reaction product, has been reported to be a potent inhibitor of ACL (Inoue et al. 1966). The inhibition is competitive with respect to ATP. Fig. 6 shows that vanadate inhibition is additive to that of ADP, suggesting that the site of the vanadate action is different from that of ADP. This was rather unexpected since according to the kinetic data mentioned above, vanadate anions, ATP and obviously ADP should compete for the common site at the catalytic domain of the enzyme.

Discussion

This report presents another enzyme the catalytic activity of which is inhibited by vanadium ions. Both pentavalent anions (mono-, decavanadate) and tetravalent

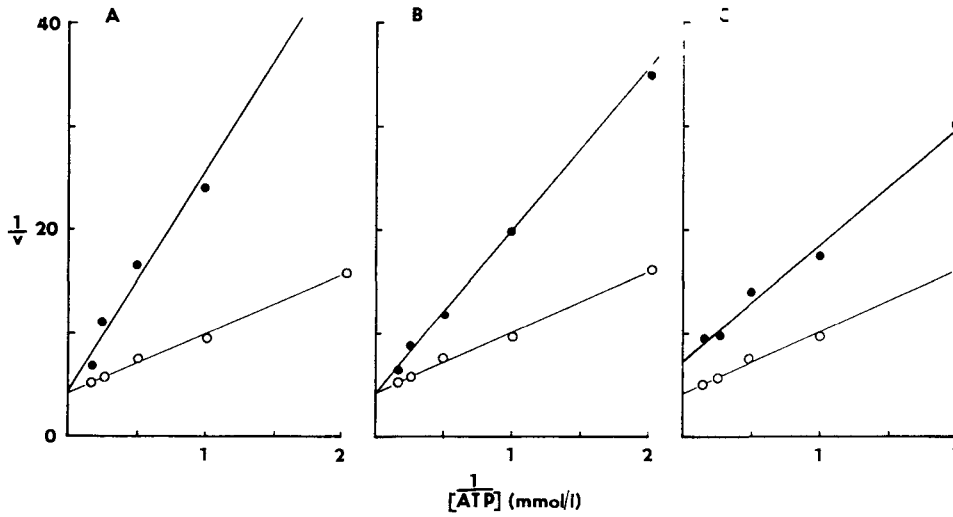

Figure 3. Inhibition by vanadium ions of ATP citrate lyase activity with respect to ATP *A*, monovanadate (10^{-3} mol/l), *B*, decavanadate (10^{-3} mol/l), *C*, vanadyl (10^{-2} mol/l) o—o, control values, •—•, + vanadium Each point represents the mean of at least three measurements

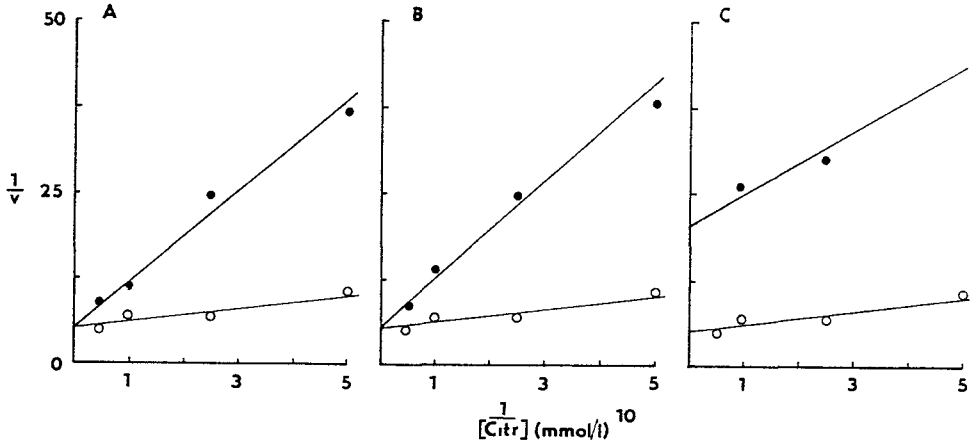

Figure 4. Inhibition by vanadium ions of ATP citrate lyase activity with respect to citrate Description as in the legend to Fig 3

vanadyl cation inhibit the enzyme The kinetic data suggest that the inhibition produced by all three species of vanadium ions with respect to ATP, substrates and

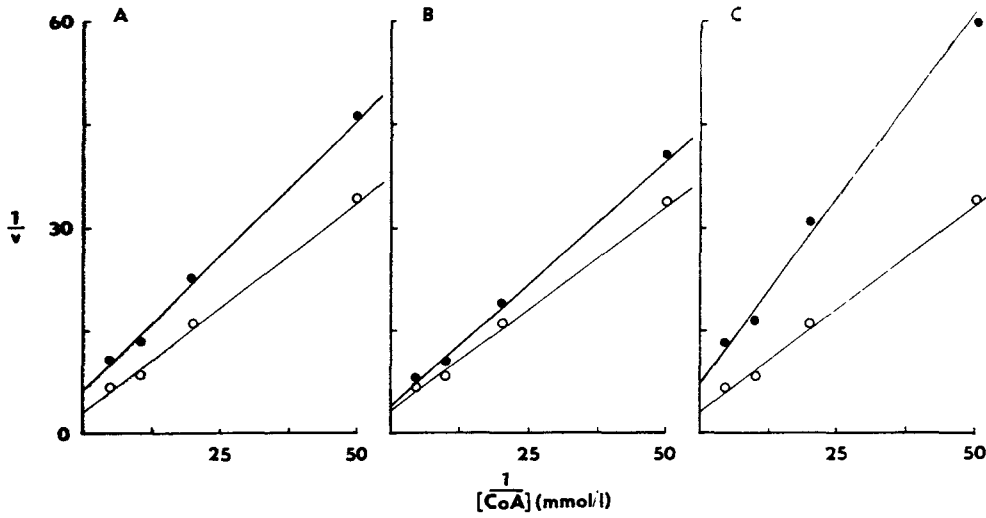

Figure 5. Inhibition by vanadium ions of ATP citrate lyase activity with respect to CoA. Description as in the legend to Fig. 3.

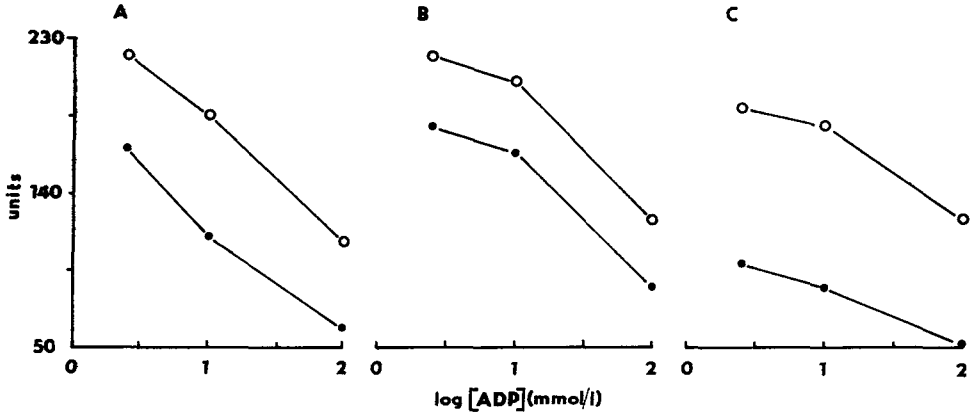

Figure 6. Inhibition of ATP citrate lyase activity by ADP in the presence of *A*, monovanadate (10^{-3} mol/l); *B*, decavanadate (10^{-3} mol/l); *C*, vanadyl (10^{-2} mol/l). o—o, without vanadium; •—•, in the presence of vanadium. Each point represents the mean of two measurements.

CoA resembles that previously found for SCS (Křivánek and Nováková 1991b). Thus the mechanism of the vanadium inhibition of both enzymes appears to be

closely related. This is not surprising if one takes into account the high degree of structural and functional similarity of the two enzymes. SCS from *Escherichia coli* possesses 69% similar residues over a 230-amino acid section (Elshourbagy et al. 1989). The catalytic mechanism is also similar in that they both involve histidine autophosphorylation and putative acyl-phosphate and acyl-CoA intermediates (Inoue et al. 1966). Our original assumption was that the enzymes possessing phosphorylated histidyl residue at their catalytic sites and forming phosphoryl-E as an intermediate, are susceptible to inhibitory action of vanadium. Attenuation of the histidyl phosphorylation might be a common mechanism by which vanadium ions inhibit some phosphoryl transfer enzymes. This view seems to be supported by our finding that histidyl phosphorylation of both SCS and ACL as well as the activity of SCS and ACL are inhibited by vanadium ions (Křivánek and Nováková 1989, 1991a,b, kinetic data presented in this paper). Several other enzymes, like glucose 6-phosphatase and 2,3-phosphoglycerate dependent phosphoglycerate mutase, containing phosphorylable histidyl residue in their molecule, are inhibited by vanadate (Climent et al. 1981) but its effect on phosphorylation of their histidyl moiety has not yet been reported. The mechanism of vanadate action on these and many other enzymes has not yet been fully elucidated.

A large body of evidence has been accumulated that vanadium ions affect phosphorylation of proteins mostly those possessing phosphorylable tyrosyl residue in their molecules (see Introduction section). It appears from our experiments that also phosphorylation of the histidyl residue may be the target for vanadium ions in a number of enzymes. This would imply that also the mechanism of alteration of their catalytic activity by vanadium ions might be the same. It has not yet been definitely proved whether suppression of the histidyl phosphorylation is due to stimulation of the (specific?) phosphatase(s) or inhibition of appropriate kinase(s). However, still another possibility may exist, namely formation of a histidyl-vanadate complex which can proceed even nonenzymatically (Rehder 1991).

From the experimental data hitherto published several possible ways in which vanadate affect enzyme activity have been put forward. (1) Vanadate oxidation of thiol groups (Benabe et al. 1987). This hypothesis has not been justified by other authors (Mendz et al. 1990; Crans and Simone 1991). (2) Binding of vanadate monomer to the binding site for phosphate functioning as an analogue of inorganic phosphate. Vanadate can form a stable trigonal bipyramidal structure analogue of phosphate which is an intermediate for E-P formation (Chasteen 1983). (3) Inhibition through formation (even nonenzymatic) of organic vanadate dead-end inhibitory complexes (Stankiewitz et al. 1987; Perceival et al. 1990). (4) Inhibition by vanadate oligomers (di- and tetra-) by the different mechanisms neither of which involves formation of the phosphate analogue (Boyd et al. 1985; Crans et al. 1990). (5) Enzymatic conversion of spontaneously formed organic vanadate compounds by enzymes which under normal conditions convert corresponding phosphate organic

compounds (Nour-Eldeen et al. 1985; Drueckhamer et al. 1989). Our data might perhaps match most closely the characteristics (2) of the above list.

Some differences between the vanadium effect on SCS and ACL may exist. It appears from our data that susceptibility to vanadate of ACL differs from that of SCS. This may be due to some interaction of vanadate with the substrates of ACL since presence of citrate plus CoA significantly depressed the inhibitory effect of vanadate on the endogenous phosphorylation of ACL whereas that of SCS was not affected by its substrate (Table 1). Also dependence of the vanadate effect on the concentration of substrate is different in both enzymes: vanadate inhibition of SCS activity was not detectable up to 20 mmolar concentration of succinate (Křivánek and Nováková 1991b). It is possible that the mechanism of vanadate action on the catalytic activity of ACL differ in some respect from that of SCS and that besides inhibition of histidine phosphorylation some additional steps in the catalytic mechanism might be involved. Vanadate at micromolar concentrations which inhibit SCS, is present predominantly as a monomer in the medium (Chasteen 1983). However, at higher concentrations (inhibition of ACL) also oligomers (namely di- and tetra-) might be the active forms of vanadate (Boyd et al. 1985). The mechanism of their action may or may not differ from that of monovanadate. However, if the portion of vanadate is bound by a component of medium (citrate) the effective vanadate concentration may still remain low, comparable with that affecting SCS.

In spite of the widely accepted hypothesis that vanadyl rather than vanadate is a biological form of vanadium (Canthely and Aisen 1979) its effects have been much less studied as compared to vanadate. Usually vanadyl has been shown to be less effective than vanadate or to exert no effects (Schmitz et al. 1982; Hayashi and Kimura 1985; Křivánek and Nováková 1988, 1989, 1991a,b). The relatively weak activity of vanadyl was demonstrated also in this paper but its efficiency was identical with that previously shown for SCS (Křivánek and Nováková 1991b), yet comparable with that of vanadate action on ACl. The mechanism of the low efficiency is not yet clear. Some possibilities have been discussed earlier (Křivánek and Nováková 1988). From the kinetic data (Figs. 3, 4) it seems that even the mechanism of the vanadyl inhibitory action on ACL might differ from that of vanadate in that it acts as a noncompetitive inhibitor with respect to both ATP and citrate. The kinetic data revealed close similarity between the vanadium action on SCS and ACL. However, other data, namely lower susceptibility of ACL to vanadate as well as dependence of the histidine phosphorylation of ACL on presence of substrate, suggest that in some respect the mechanism of the vanadium ions inhibition on the two enzymes may be different. It may be more complex and could not be fully accounted for by mere inhibition of the histidine phosphorylation. Thus our hypothesis that this might be a common mechanism by which vanadium ions inhibit phosphoryl transfer enzymes is not justified.

Present experiments extend the list of enzymes the activity of which is affected by vanadium ions. Possible biological role of this vanadium action is difficult to estimate. Taking into account that only relatively high concentration of vanadate are effective one can hardly expect some physiological (regulatory) implications of inhibition of ACL by vanadate. The possibility remains that at least during vanadate intoxication such an influences on metabolism of the living cells might take place.

Acknowledgements. I thank Dr. M. Havránek for preparation of [γ-^{32}P]ATP and Mrs L. Nováková for skilful technical assistance.

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Final version accepted February 26, 1994