A Novel Effect of Vanadium Ions: Inhibition of Succinyl-CoA Synthetase

J. KŘIVÁNEK and L. NOVÁKOVÁ

Institute of Physiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 142 20 Prague, Czechoslovakia

Abstract. Effect of vanadate and vanadyl ions on the ATP-dependent succinyl-CoA synthetase (A-SCS) solubilized by Lubrol-PX from the rat brain mitochondria was tested. Vanadate added to the assay medium at 10^{-5} mol.l^{-1} and 10^{-4} mol.l^{-1} concentrations inhibited the enzyme activity by about 50 % and 94 %, respectively. When the enzyme was solubilized from the mitochondria preincubated with 10^{-4} mol.l^{-1} and 10^{-3} mol.l^{-1} vanadate, the residual inhibitions were 55 % and 100 % respectively. The vanadyl cation also induced inhibition of the A-SCS activity but the effect was less expressed. At 10^{-4} mol.l^{-1} concentration only 20 % inhibition was achieved. The A-SCS solubilized from the mitochondrial subfractions (perikaryal, light and heavy synaptosomal) differed neither in the activity of A-SCS nor in the susceptibility toward action of vanadium ions. A strong dependence of the vanadate inhibition on the concentration of succinate was observed. The above effect (50 % inhibition) could be demonstrated only at saturating concentration of succinate (50 mmol.l^{-1}). The mechanism of vanadium ions action as well as differences between vanadate and vanadyl ions effects are discussed.

Key words: Vanadate — Vanadyl ions — Succinyl-CoA synthetase — Brain mitochondria

Introduction

We have shown previously that both the vanadate anion (VO_3^-; $H_2VO_4^-$) and the vanadyl cation (VO^{2+}, suppress phosphorylation of the mitochondrial protein

Abbreviations. A-SCS, ATP-dependent succinyl-CoA synthetase [succinate: CoA ligase (ADP), EC 6.2.1.5]; G-SCS, GTP-dependent succinyl-CoA synthetase [succinate: CoA ligase (GDP), EC 6.2.1.4.]; DTT, dithiothreitol; EGTA, Ethyleneglycol-*bis*- (*β*-amino-ethyl ether) N, N-tetraacetic acid.

of Mr 34.5 kD (Křivánek 1988; Křivánek and Nováková 1989a). More recently we have identified this protein as an alpha-subunit of succinyl-CoA synthetase [succinyl thiokinase; succinate: CoA ligase (ADP), EC 6.2.1.5 and (GDP), EC 6.2.1.4] (Křivánek and Nováková 1989b). The enzyme catalyzes reversible formation of succinyl-CoA from succinate, CoA and ATP (GTP). Since the phosphorylated form of this enzyme is intermediate in the catalytic reaction the effect of vanadium ions on the activity of this enzyme solubilized from the rat brain mitochondria was tested. A potent inhibitory effect of vanadate was found. Vanadyl cation also inhibited the enzyme activity but the effect was less markedly expressed.

Materials and Methods

Brain of male hooded rats (Long-Evans strain, 180—200 g b.w.) was the source of mitochondria.

Vanadate ($NaVO_3$) was from Aldrich-Chemie (Steinheim, FRG), vanadyl sulfate was supplied by Jansen Chimica (Beerse, Belgium). ATP, disodium salt (vanadate free) was from Boehringer (Mannheim, FRG). Coenzyme A, sodium salt and DTT were from Sigma Chemicals (St. Louis, USA). Other chemicals were of purity grade.

Whole brain mitochondria were prepared by the procedure of Jones and Matus (1974). To obtain total (perikaryal plus synaptosomal) mitochondria, the crude mitochondrial fraction was lysed in 5 mmol.l^{-1} Tris-HCl buffer, pH 8.1 and separated from myelin and membrane fractions by combined flotation sedimentation — density gradient centrifugation. The final mitochondrial pellet was washed with 4 mmol.l^{-1} imidazole buffer, pH 7.5 and stored as a suspension in a 1:1 mixture of imidazole buffer-glycerol at −20 °C.

Perikaryal and two synaptosomal mitochondrial fractions were prepared by the procedure described by Lai and Clark (1979) based on centrifugation of lysed mitochondria on the Ficoll gradients.

Preincubation of mitochondria: Mitochondrial suspensions were incubated under conditions as used previously for testing the effect of vanadate on endogenous phosphorylation of proteins in mitochondrial suspensions (Křivánek 1988). Sixty second preincubation with 20 μmol.l^{-1} ATP was followed by addition of 100 μl of mitochondrial suspension (about 1 mg protein) with vigorous shaking for the next 15 s in a total volume of 500 μl medium consisting of (in mmol.l^{-1}): Tris-HCl buffer, pH 7.4 (50); $MgCl_2$, (10); EGTA, (2) in the presence or absence of vanadate or vanadyl in concentrations indicated in the graphs. After cooling the same volume of 1 % Lubrol-PX reagent was added to the reaction mixture as mentioned below.

Solubilization of mitochondria: Under continuous stirring the mitochondrial suspension was mixed with the Lubrol-PX reagent to get the final concentration of 0.5 % Lubrol in 0.24 mol.l^{-1} Tris-HCl, pH 7.4, 9 % (w/v) sucrose (Tsakiris 1984). After keeping the mixture at 4 °C overnight the soluble fraction was obtained by spinning the mixture at 150,000 × g for 90 min.

The activity of SCS was determined by measuring the rate of change in absorption at 235 nm (Cha 1969), using Phillips UV/VIS spectrophotometer of the PU 8700 series allowing to work at high absorbancies (up to 3.0 absorbance unit). The standard reaction mixture consisted of 50 mmol.l^{-1} Tris-succinate pH 7.4, 10 mmol.l^{-1} $MgCl_2$, 0.1 mmol.l^{-1} ATP, 0.1 mmol.l^{-1} coenzyme A. After 3 min preincubation at 30 °C the reaction was started by adding an aliquot of the Lubrol extract of mitochondria. The enzyme activity was expressed as nkat. mg prot.$^{-1}$ ± SEM.

Table 1. K_m values for SCS substrates. The Lubrol-PX extracts of the brain mitochondria were incubated as described in Materials and Methods. Other substrates were used at near saturating concentrations. The data for the heart and *E. coli* SCS are from the work of Bridger (1974).

Substrate	K_m (mol/l)		
	Brain mitochondria	*E. coli*	Heart
ATP	1.7×10^{-5}	2.0×10^{-5}	—
GTP	—	—	1.0×10^{-5}
CoA	5.6×10^{-5}	1.5×10^{-6}	2.0×10^{-5}
Succinate	1.6×10^{-3}	1.0×10^{-4}	8.0×10^{-4}

Proteins were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Student's *t*-test was used for the statistical evaluation of the results.

Results

Effect of vanadate on SCS. Since A-SCS prevails in the brain (Weitzman et al. 1986) it was characterized first. The apparent K_m values of our preparation for several substrates at near-saturating concentrations of other substrates is presented in Table 1. With ATP as a substrate, K_m approaches that of *E. coli* SCS which is A-form. For succinate and CoA, however, the K_m appears to be closer to that of pig-heart SCS (G-form). The A-form from brain seems to somewhat differ from the A-form from other tissue tested.

At saturating concentration of its substrates SCS activity is inhibited by vanadate at 10^{-4} mol.l^{-1} concentration. As shown in Figure 1, the inhibitory effect of vanadate is about twice that exerted on the phosphorylation of the SCS alpha-subunit. At vanadate concentration of 10^{-5} mol.l^{-1}, the activity of SCS is inhibited by about 50 % while phosphorylation of the alpha-subunit is inhibited only by 26 %. At 10^{-4} mol.l^{-1} the respective inhibitions were 94 % and 65 %. The inhibition appears to be of a competitive nature with respect to ATP when using saturating concentration of succinate (50 mmol.l^{-1}). A similar picture is obtained with CoA as a substrate (not shown). In contrast to the above results practically no inhibition of SCS by vanadate could be observed at lower concentrations of succinate (1.0; 2.5; 12.5 mmol.l^{-1}). Inhibition by vanadate (57 % and 58 % — Fig. 3) only appeared at 50 and 75 mmol.l^{-1} succinate

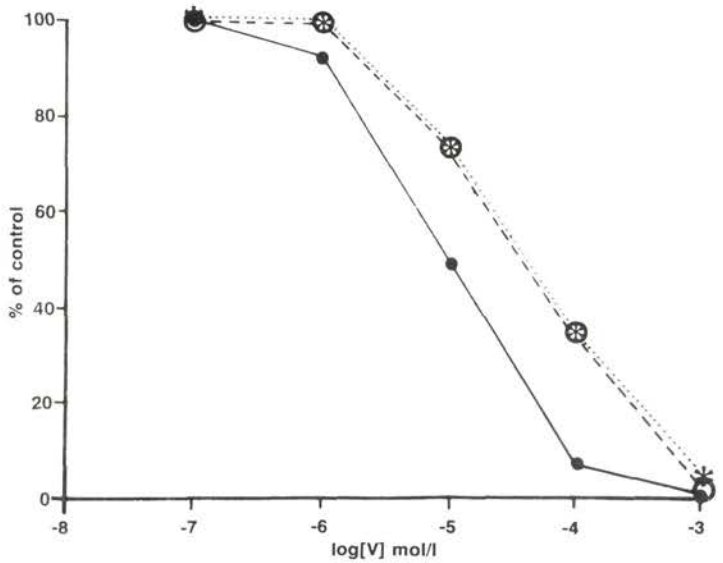

Fig. 1. The effects of vanadate on the activity of SCS (● ●), phosphorylation of the mitochondrial 34 kD protein (○ ○) and the autophosphorylation of the alpha-subunit of SCS (* *). Each point represents the mean of at least four measurements ± SEM.

concentrations respectively. It is obvious, therefore, that the presence of succinate at some critical concentration is crucial for vanadate to exert its inhibitory action.

Effect of vanadyl on SCS. It is generally accepted that the tetravalent, reduced form of vanadium — vanadyl is the biologically active form (Cantley and Aisen 1979; Macare et al. 1980). A comparison of the effects of vanadate and vanadyl on SCS activity is illustrated in Fig. 4. Like in many other systems hitherto tested (see Discussion), vanadyl is a much less potent inhibitor of SCS activity than vanadate added to the assay medium. At 10^{-4} mol.l^{-1} concentration vanadyl inhibited SCS by only 19 % whereas vanadate inhibited the enzyme activity almost completely (see above).

Effect of vanadium ions on SCS of mitochondrial subfractions. Steiner and Smith (1981) have described the heterogeneity of the brain mitochondria with respect to ATP- and GTP-dependent endogenous phosphorylation of the SCS alpha-subunit. Perikaryal mitochondria contained equal amounts of the ATP- and GTP-dependent endogenous phosphorylation of the SCS alpha-subunit by [gamma-^{32}P]ATP, whereas light synaptic mitochondria (SM1) contained almost exclusively the ATP-dependent form. The heavy mitochondrial fraction of

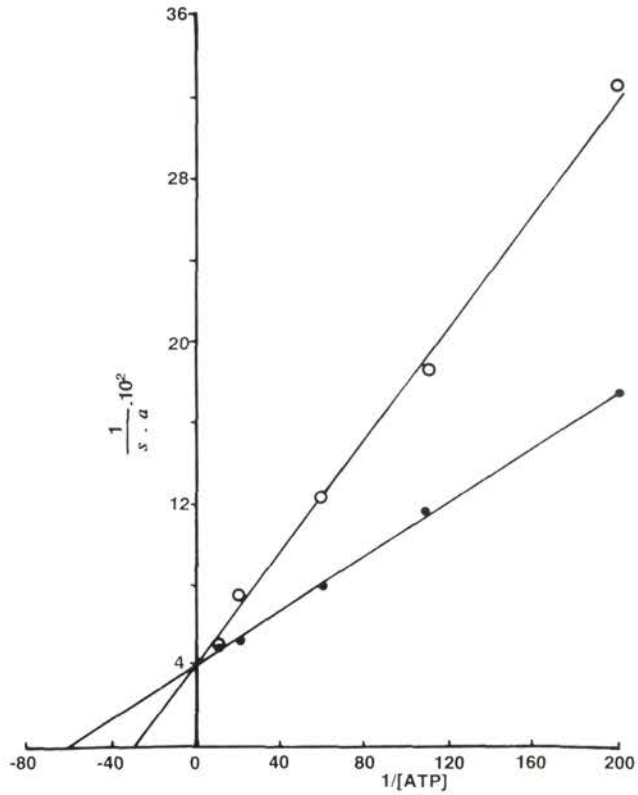

Fig. 2. Lineweaver-Burk plot of the dependence of SCS activity on ATP concentration. Filled symbols: ATP (mmol/l); empty symbols: ATP + 10^{-5} mol . l^{-1} vanadate. Each point is the mean of five measurements.

synaptosomes (SM2) contained the ATP-dependent form but also small amounts of the GTP-form. In our experiments no difference in ATP-dependent activities of SCS could be observed among the three mitochondrial subfractions (Fig. 5). The basal activity of the A-form of SCS in M, SM1 and SM2 were 1.58 + 0.90; 1.67 + 0.09; and 1.40 + 0.13 units, respectively. The respective values for inhibitions by 10^{-5} mol . l^{-1} vanadate were 52 %; 55 %; and 56 %, respectively. Thus, in contrast to the heterogenous phosphorylation of the SCS alpha-subunit we were not able to show such a variability. However, the activity of the G-form of SCS was not measured so that also the ratio of the two forms may have differed in our preparations.

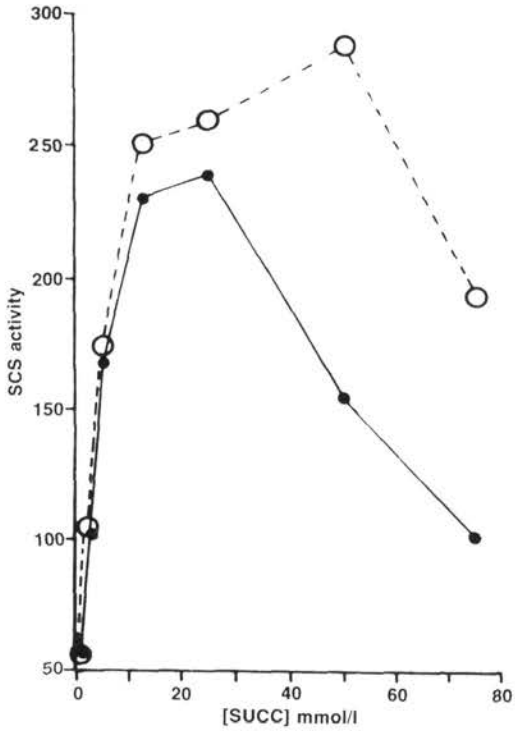

Fig. 3. The dependence of the inhibitory action of vanadate on SCS on succinate concentration. ○—○, in the absence of vanadate; ●—●, SCS activity in the presence of 10^{-5} mol.l^{-1} vanadate. SCS activity is expressed in nkat.mg prot.$^{-1}$. Each point represents the mean of at least three measurements ± SEM.

Preincubation of mitochondria with vanadium ions. Less pronounced inhibition of SCS by vanadyl as compared to that by vanadate could be also observed when the mitochondria had been preincubated in the presence of vanadate or vanadyl ions (Fig. 6). The basal activities are close to those of SCS solubilized from non-preincubated mitochondria. However, the sensitivity toward the effect of vanadium ions is somewhat less expressed. Thus 10^{-4} mol.l^{-1} vanadate added to the assay medium almost completely inhibited SCS but when added to the preincubation medium, the solubilized enzyme was inhibited only by 46 %. This might be due at least partially to the release of vanadium ions from their binding sites during the treatment of mitochondria after preincubation (solubilization with Lubrol, centrifugation) and/or to a poor access to the site of their action in the inner membrane of intact mitochondria where SCS resides (Leh-

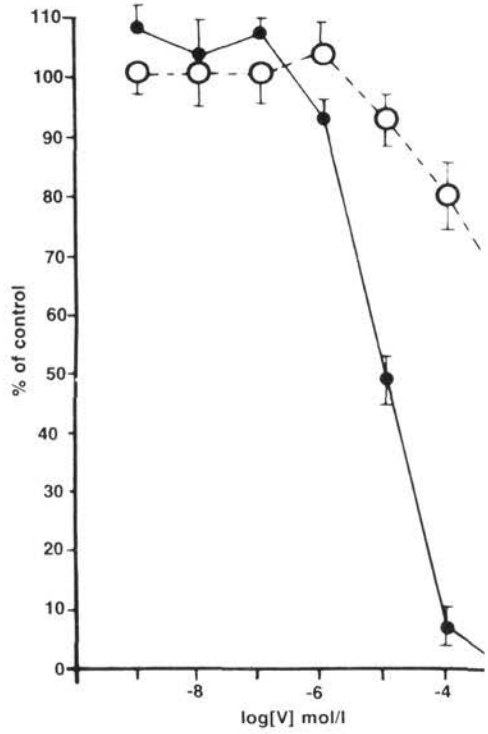

Fig. 4. The effect of vanadate (●—●) as compared with that of vanadyl (○—○) added to the assay medium on the activity of SCS solubilized from the mitochondria by Lubrol. Each value represents the mean of at least three measurements ± SEM.

ninger 1975). This assumption is supported by a less striking effect of vanadium ion on SCS of the preincubated mitochondria than on the solubilized enzyme (Fig. 7). Under these conditions (preincubation of the mitochondrial subfractions with vanadium ions) the inhibitory effect of vanadyl was again less expressed than that of vanadate. However, the differences between vanadyl and vanadate influences were much less striking than when vanadium ions had been added directly to the assay medium. This is illustrated in Fig. 7, where the ratio of the inhibitory effect of 10^{-4} mol.l^{-1} vanadate is compared to that of 10^{-4} mol.l^{-1} vanadyl added to the assay medium (4.5), with the same ratio of inhibitions induced by vanadium ions added to the preincubated media (1.5). If the difference between the direct effect and that observed after preincubation was due to the release of at least a part of the vanadium ions as suggested above,

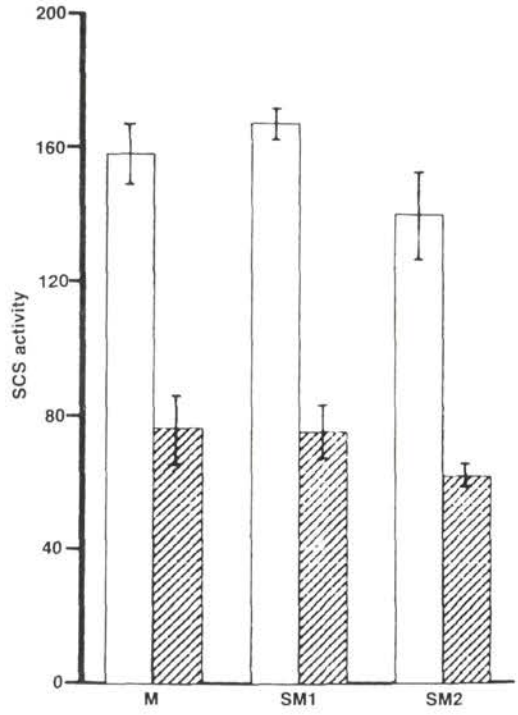

Fig. 5. Effect of vanadate on the activity of SCS solubilized by Lubrol from three populations of the brain mitochondria: perikaryal (M), light synaptosomal (SM1), heavy synaptosomal (SM2). Empty columns: control activity; hatched columns: activity in the presence of 10^{-5} mol.l^{-1} vanadate added to the assay medium. Each value represents the mean of three measurements ± SEM.

the different dissociation constants for vanadate and vanadyl should be considered.

Discussion

The results presented in this communication show a novel effect of vanadium ions — inhibition of the activity of succinyl-CoA synthetase. In our previous experiments examining the effects of vanadate on protein phosphorylation in brain mitochondria, ATP was used as a donor of the gamma-P (Křivánek 1988; Křivánek and Nováková 1989a). This was the case also in the experiments demonstrating the identity of the mitochondrial 34 kD protein with the alpha-subunit of SCS (Křivánek and Nováková 1989b). Thus this was the A-form of

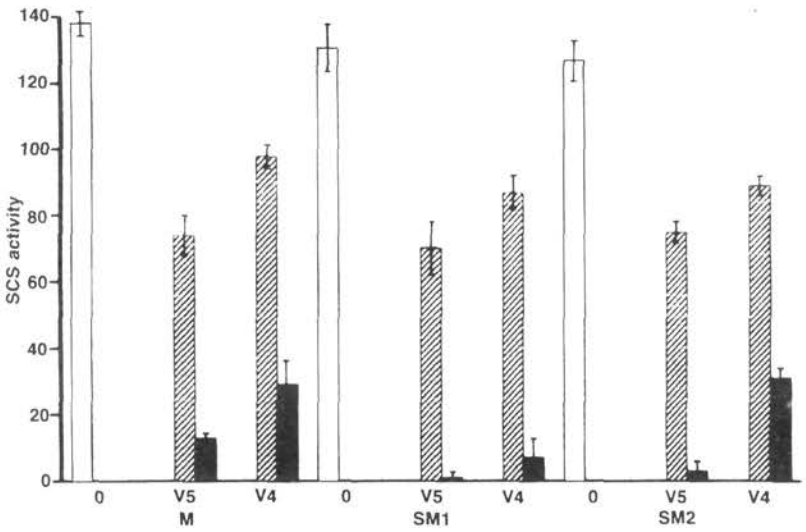

Fig. 6. The activity of SCS solubilized by Lubrol from three mitochondrial populations preincubated under conditions of endogenous protein phosphorylation (Křivánek 1988) in the absence (empty columns), or presence of vanadate (V5) or vanadyl (V4) at 10^{-4} mol.l^{-1} (hatched columns) and 10^{-3} mol.l^{-1} (filled columns). For other symbols see the legend to Fig. 5. Each value represents the mean of at least three measurements ± SEM.

SCS the alpha-subunit phosphorylation of which was shown to be inhibited by vanadium ions. In contrast with most vertebrate tissues, in which G-form predominates or even occurs exclusively (Weitzman et al. 1986; Hansford 1973; McClellan and Ottaway 1980; Hamilton and Ottaway 1981; Bridger 1974), in the brain of adult vertebrates the A-form is the major type of SCS. The reported A-form/G-form ratios in the brains of pigeon, chick and rat are 2.22; 7.14; and between 1.67 and 3.85, respectively (Weitzman et al. 1986; Hamilton and Ottaway 1981).

A potent inhibitory effect of vanadate on SCS is demonstrated in this paper. At 10 micromolar concentration vanadate inhibited the enzyme activity by about 50 %. Since the phosphoenzyme is an intermediate in the reaction mechanism the effect is obviously due to suppression of autophosphorylation of the alpha-subunit of SCS as shown recently (Křivánek 1988; Křivánek and Nováková 1989a, b). However, SCS activity is inhibited much more than phosphorylation of its alpha-subunit (Fig. 1). Vanadate at 10^{-5} mol.l^{-1} concentration exhibited only 24 % inhibition of phosphorylation. This difference may

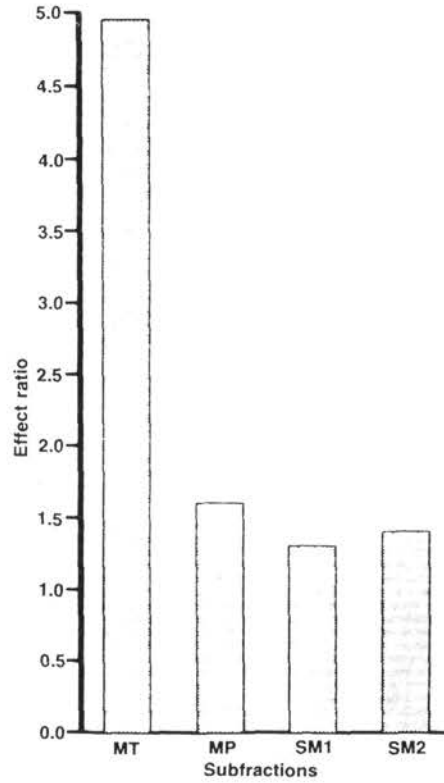

Fig. 7. Differences between the effects on the SCS activity of vanadate added to the assay medium containing Lubrol—solubilized SCS ("direct effect") and that exerted by vanadate added to the medium in which intact mitochondria had been preincubated, followed by solubilization with Lubrol ("persisting effect"). The values are the "direct effect" to "persisting effect" ratios in the three mitochondrial subfractions (MP, perikaryal; SM1, light synaptosomal; SM2, heavy synaptosomal) and unfractioned (total) mitochondria (MT).

simply reflect the fact that there is no close quantitative correlation between the degree of phosphorylation and the catalytic activity of the SCS. Effect of vanadate on the SCS alpha-subunit phosphorylation was demonstrated on relatively intact mitochondrial preparations, whereas its action on SCS activity was tested on the enzyme solubilized from mitochondria by Lubrol as the activity of the intact mitochondria is very low (Hansford 1973). It seemed reasonable to assume that the accessibilities of the binding sites for vanadate differ in the two preparations. However, this is unlikely because the extent of the vanadate inhibition of phosphorylation of the alpha-subunit of the partially

purified, soluble commercial preparation of SCS is exactly the same as in intact mitochondria (Křivánek and Nováková 1989b). Vanadate might affect still another step(s) in the activity of SCS. This view is supported by the dependence of the vanadate effect on the succinate concentration (Fig. 2).

Considering the generally accepted view that vanadyl is the biologically active form of the intracellular vanadium (Cantley and Aisen 1979; Macara et al. 1980) and that there is a carrier for vanadate (Werdan et al. 1980) we have attempted to explain the difference in the efficiency of the two ions when added to the intact cells or particulate systems by differences in the accessibility of their sites of action (Křivánek and Nováková 1988). It was assumed that this is more favorable for vanadate (existence of its carrier) than for vanadyl (intracellular location). The present results do not support this idea since the potency of vanadate is higher even with a solubilized enzyme preparation. Moreover, in contrast to vanadate, vanadyl inhibits both the SCS alpha-subunit phosphorylation (Křivánek 1988) and its enzyme activity to the same extent, though still much less efficiently than vanadate does. This is another, though indirect, evidence for the above assumption that vanadate affects SCS activity in a more complex way than does vanadyl, the action of which can be fully explained by the inhibition of phosphorylation of the SCS alpha-subunit. These results suggest that the two types of vanadium ions do not exert their effects by the same mechanism. Different redox state and/or electrical charge of these ions may be responsible for the difference in their action. The quantitative difference between vanadyl and vanadate is much less striking when SCS activity is determined on the enzyme solubilized from the mitochondria preincubated with one of these ions (Fig. 7). Both the accessibility of the binding site and the susceptibility of the vanadate and vanadyl bonds to the treatment may differ, following the preincubation of mitochondria.

Any possible physiological relevance of the vanadate and vanadyl effects hitherto described, including those demonstrated in the present paper, is obscure as yet. In spite of a huge amount of work invested into the research into the biological actions of vanadium ions no unambiguous evidence has yet been provided for their possible regulatory role in the living cells. The control of an important enzyme in both pyruvate and ketone body metabolism such as SCS (Jenkins and Weitzman 1986) is significant for the cellular metabolism and function. To what extent, if at all, vanadium ions could participate in the regulation of these metabolic pathways, be it under physiological or pathological conditions, is difficult to judge. The present results just extend the spectrum of the biochemical effects of vanadium ions.

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