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# The 34 kDa mitochondrial protein, phosphorylation of which is inhibited by vanadate, is the α-subunit of succinyl-CoA synthetase

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Our previous characterization of the 34 500 Da protein, which occurs exclusively in mitochondria and the phosphorylation of which is inhibited rather than stimulated by vanadate, suggested that it might be the α-subunit of succinyl-CoA synthetase (SCS). In the present communication we show that in a commercial preparation of SCS there is only one band (34 500 Da) which is phosphorylated under endogenous phosphorylation conditions in vitro. The relative molecular weight of this band, sensitivity of its phosphorylation to the inhibitory action of both vanadate and vanadyl as well as dose response curves are identical to those described for the mitochondrial '34 kDa' protein. This is the first demonstration of an inhibitory effect of vanadium ions on the autophosphorylation of the α-subunit of SCS.

Succinyl CoA synthetase; Protein, 34 kDa; Protein phosphorylation; Vanadate; Vanadyl; Mitochondrion

## 1. INTRODUCTION

The capability of vanadate to affect phosphorylation of proteins from various sources has been demonstrated in many laboratories [1]. Also the enhancement of endogenous phosphorylation of brain proteins has been reported [2–4]. However, up until now, only a stimulatory effect on protein phosphorylation, be it due to stimulation of appropriate kinases or due to inhibition of protein phosphatases, has been demonstrated. Recently we have found, in brain and in other mammalian tissues, a peptide occurring exclusively in mitochondria, the endogenous phosphorylation of which was inhibited rather than stimulated by both vanadate and vanadyl [4,5]. All the characteristics of this protein and its phosphorylation, including the relative molecular weight, acid lability of the phosphate bond (P-N linkage to some of the basic amino acid), suggest that the mitochondrial 34 kDa protein might be identical to the α-subunit of succinyl CoA synthetase (succinic thiokinase; succinate: CoA ligase EC 6.2.1.4). The present experiments show that autophosphorylation of the α-subunit of a commercial preparation of this enzyme is indeed inhibited by both vanadate and vanadyl ions.

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## 2. MATERIALS AND METHODS

Cerebral cortex from male hooded rats (Long-Evans strain) of 180–200 g body wt was the source of the mitochondria used.

Partially purified succinyl-CoA synthetase preparation from porcine heart was a product (S 4755) of Sigma (St. Louis, MO, USA). Sodium vanadate ($Na_3VO_4$) was from Fischer Co. (Munchen, FRG). Vanadyl sulfate was supplied by Jansen Chimica (Beerse, Belgium). [$\gamma$-$^{33}$P]ATP (spec. act. 1.5 TBq mmol$^{-1}$) was prepared by Dr M. Havránek from The Institute of Nuclear Biology and Radiochemistry, Czechoslovak Academy of Sciences, Prague. All reagents for polyacrylamide gel electrophoresis were from Serva (Heidelberg, FRG). Dithiothreitol and 2-mercaptoethanol were from Sigma.

Mitochondria including the two synaptosomal species were prepared by a flotation-density gradient centrifugation of lysed crude mitochondria [6].

The commercial preparation of SCS was dissolved in 30 mM Tris-HCl buffer, pH 7.4, to the concentration of 2 mg·ml$^{-1}$ (20 U·ml$^{-1}$).

The endogenous phosphorylation assay was performed in a medium of a total volume of 50 μl consisting of (in mM): Tris-HCl buffer, pH 7.4 (50); $MgCl_2$ (10); dithiothreitol (1); EGTA

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(2);. [$\gamma$-$^{33}$P]ATP (0.02; 4000 cpm per pmol); about 100 $\mu$g mitochondrial proteins or 10 $\mu$g SCS. Vanadate and vanadyl as indicated in the legends to the figures. After 60 s preincubation with enzyme preparation or suspension of mitochondria at 30°C, the incubation was started by addition of [$^{33}$P]ATP followed by vigorous shaking for 20 s. Phosphorylation was stopped by Laemmli's sample solution [7] and heating in boiling water for 3 min. One-dimensional slab gel electrophoresis was performed by the method of Laemmli [7]. 50 $\mu$l aliquots, i.e., 50 $\mu$g of mitochondrial proteins or 5$\mu$g SCS preparation were loaded onto the gel. 4% and 10% acrylamide were used for stacking and separatory gels, respectively. After staining, destaining [8] and drying the gels were exposed to the FOMA Medic Rapid X-Ray film. For quantitative radioactivity measurements, the bands of interest were dissected out of the gels, tranferred into scintillation cocktail based on toluene and counted by liquid scintillation spectroscopy. The data were corrected by blanks, i.e. by subtracting the radioactivity from gel pieces of comparable size revealing no radioactivity on the autoradiograms.

## 3. RESULTS

Although the commercial preparation of SCS is only partially purified, as seen in fig.1, there is only one band, the $\alpha$-subunit, which is phosphorylated under conditions of endogenous phosphorylation (without added kinase). The presence of kinase in this preparation cannot be excluded, however, as the $\alpha$-subunit of SCS is known to be an autophosphorylative kinase it is most likely that in the present experiments phosphorylation of the 34 500 Da band is also the result of autophosphorylation. The $R_f$ and $M_r$ values of SCS are identical with those of the mitochondrial 34 kDa protein. When SCS is incubated together with the mitochondrial suspension, the resulting radioactivity is almost equal to the sum of the radioactivities of SCS and the 34 kDa mitochondrial protein determined separately (not shown).

Both vanadate and vanadyl exert qualitatively and quantitatively comparable effects on the phosphorylation of both the mitochondrial 34 kDa protein and the $\alpha$-subunit of SCS. Millimolar vanadate completely blocked phosphorylation of the two bands (fig.2). Dose-dependent curves of inhibition by vanadate are quite identical (fig.2). Also, $10^{-3}$ M vanadyl exerts the same inhibitory effect on both the $\alpha$-subunit of SCS and the 34 kDa mitochondrial protein (fig.3). Sensitivitity to $Mg^{2+}$ is also identical for phosphorylation of the 34 kDa and $\alpha$-subunit of SCS (not shown).

A close relationship or even identity between parameters tested in the two preparations provides more direct evidence for the assumption that this is the $\alpha$-subunit of succinyl-CoA synthetase, the autophosphorylation of which is inhibited by both vanadate and vanadyl.

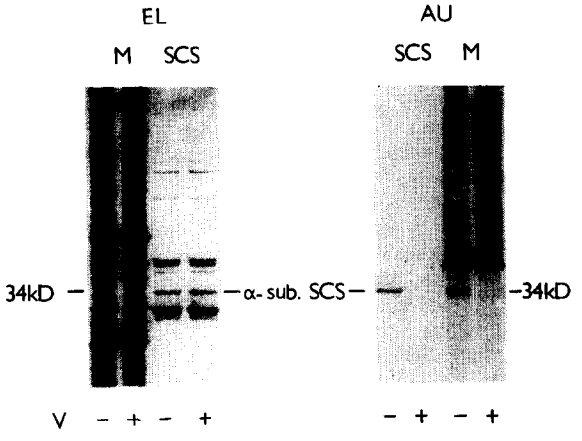


Fig.1. Effect of vanadate on endogenous phosphorylation of the rat cerebral cortex mitochondria as well as on that of a commercial preparation of succinyl-CoA synthetase. EL, electropherograms; AU, autoradiograms; M, suspension of the cerebral cortex mitochondria; SCS, commercial partially purified preparation of SCS from Sigma; V, $10^{-3}$ M vanadate.

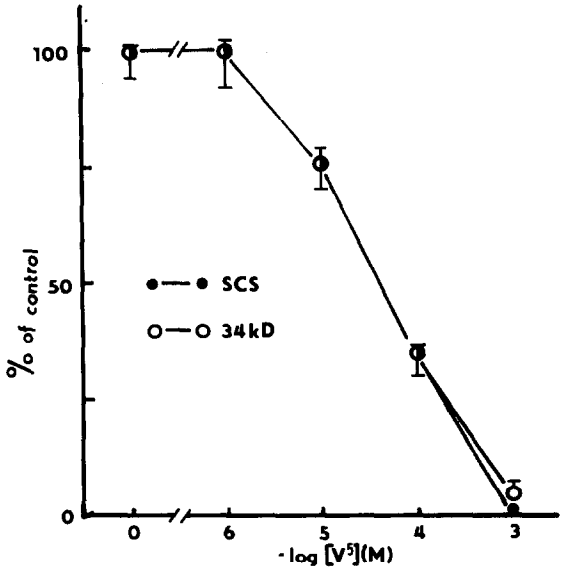


Fig.2. Dose-response curves to vanadate of endogenous phosphorylation of the mitochondrial 34 kDa protein (○—○) and that of $\alpha$-subunit of SCS (●—●). Each value represents the mean of at least four measurements ± SE.

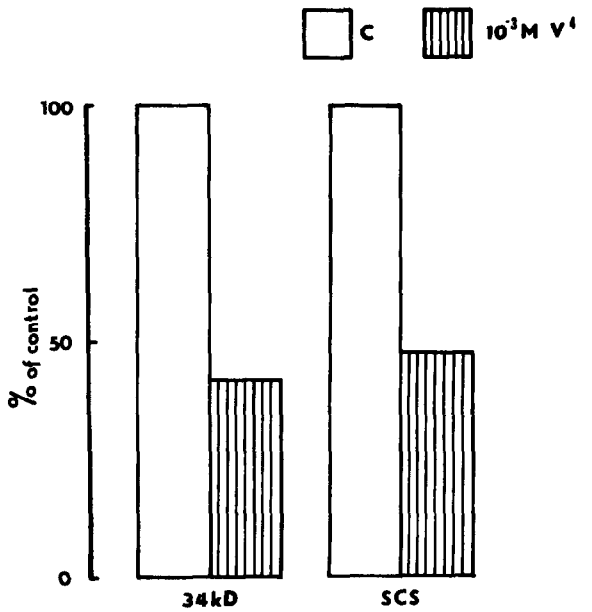


Fig.3. Effect of $10^{-3}$ M vanadyl on endogenous phosphorylation of the mitochondrial 34 kDa protein and on that of the α-subunit of SCS. (Open columns) controls; (shaded columns) $10^{-3}$ M vanadyl; 34 kDa, mitochondrial 34 kDa protein; SCS, α-subunit of succinyl-CoA synthetase. Each value represents the mean of at least three measurements.

## 4. DISCUSSION

Present results show that autophosphorylation of the α-subunit of SCS is inhibited by both vanadate and vanadyl. The entire mechanism of this effect is not definitely known yet. A direct effect on kinase activity rather than stimulation of a tentative phosphatase activity has been suggested [5]. Phosphorylation of the 34 kDa mitochondrial protein has been shown to be affected by neither of the following agents or treatment: cyclic AMP, Ca-calmodulin complex, Ca-phospholipid, shift of pH from 6.6 to 8.1 [4]. Thus the autophosphorylation of SCS represents a special type of kinase. Influence of vanadate on the tyrosine protein phosphorylation is perhaps the most detailed studied effect of this ion on the protein phosphorylation processes. Despite the existing hypothesis, no definite conclusion concerning the mechanism of action has been provided as yet [1].

Two forms of SCS, ATP-dependent (A-form) and GTP-dependent (G-form), exist in the brain [10,11]. Since in the present experiments ATP is used as phosphate donor, the results concern the A-form. As both vanadate and vanadyl affect phosphorylation of the mitochondrial 34 kDa protein from $-^{32}$P/GTP in a similar manner as that from ATP (unpublished) it is obvious that phosphorylation of the α-subunit of both forms of SCS is equally sensitive toward the inhibitory action of vanadium ions.

Since the phosphorylated form of SCS appears to be an intermediate in the catalytic mechanism [9] it is conceivable to propose that also the activity of SCS will be inhibited. In fact our preliminary results show that vanadate indeed inhibits the activity of SCS from the brain mitochondria. SCS may play an important role not only in the energy generating mechanism in the tricarboxylic acid cycle but may also control the metabolism of ketone bodies [10], particularly in the brain. Vanadium ions might thus affect an important regulatory site of the cell metabolism. A possible physiological relevance of this effect, like most of the numerous vanadate biochemical actions, remains to be elucidated.

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