

ATP-citrate lyase is another enzyme the histidine phosphorylation of which is inhibited by vanadate

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We have recently shown that phosphorylation of histidine residue of the α -subunit of the succinyl-CoA synthetase is inhibited by both vanadate and vanadyl. To assess the universality of this inhibition, we have estimated the effect of vanadate on the phosphorylation of another enzyme ATP-citrate lyase, prepared from rat liver. This enzyme contains histidine as the only amino acid with an acid-labile (P–N) phosphate bond. The 67% inhibition of endogenous phosphorylation by 1 mM vanadate disappeared after cleavage of the acidic P–N bond of histidine with acidic sample solution. The remaining 33 per cent radioactivity was due to labelling of the acid-stable phosphoamino acids (P-serine and P-threonine), the phosphorylation of which was not affected by vanadate. The dose-response curve for vanadate inhibition closely resembles that shown previously for inhibition of phosphorylation of histidine in the succinyl-CoA synthetase. The results suggest that the action of vanadate on histidyl phosphorylation is a more general effect (like its influence on phosphorylation of the protein-bound tyrosine).

Vanadate; ATP-citrate lyase; Histidine phosphorylation

1. INTRODUCTION

Vanadium is a trace element essential for the growth and normal existence of living cells. In its biologically active forms, pentavalent vanadate (VO_3^{3-} , $\text{H}_2\text{VO}_4^{4-}$) or tetravalent vanadyl cation (VO^{2+}), vanadium affects many biochemical processes, including protein phosphorylation [1]. It is not clear which of these processes, if any, are linked to the putative physiological (regulatory) or toxic actions of vanadate. Proteins, the phosphorylation of which is affected by vanadate, contain tyrosine in their molecule. It is this amino acid the phosphorylation of which is stimulated by vanadate [2–6]. Less data are available concerning the effect of vanadate on the phosphorylation of other proteins. An example is cAMP protein kinase (especially its catalytic subunit) which has been reported to be stimulated by vanadate [7,8].

More recently we have observed in the rat brain mitochondria a single protein band the endogenous phosphorylation of which was markedly suppressed by vanadate and to a lesser extent by vanadyl. The band was later identified as the α -subunit of the succinyl-CoA synthetase (SCS) [9,10], the activity of which is inhibited by vanadate and vanadyl [11]. The question arises whether this vanadate-effect is specific for this enzyme, or is rather an example of a more general action, i.e. inhibition of phosphorylation of the protein-bound histidine. To decide between these possibilities

we chose another enzyme containing histidine, ATP-citrate lyase (CL). This enzyme is a tetramer (M_r 440 kDa) of 4 apparently identical subunits. The phosphorylatable histidine resides at the catalytic site while serine is bound to 2 sites (A and B) and threonine on the B₁ site of the regulatory subunit [12–14]. If only the histidine phosphorylation is inhibited by vanadate, then under normal phosphorylation conditions vanadate (1 mM) should elicit not complete but partial inhibition of phosphorylation (as is the case with SCS containing only histidine). After mild acid hydrolysis removing P from the P–N bond of histidine, no inhibition should be observable, and the remaining radioactivity should reflect the radioactivity of serine and threonine phosphorylation unaffected by vanadate.

2. MATERIALS AND METHODS

Liver of the hooded rats (Long–Evans strain) fasted for 2 days and re-fed a fat-free, carbohydrate-rich diet [15] was the source of citrate lyase.

Polyethyleneglycol 6000 was supplied by Serva (Heidelberg, Germany) and so were all reagents for polyacrylamide gel electrophoresis. EGTA, dithiothreitol, Tris, 2-mercaptoethanol, and DEAE-cellulose were obtained from Sigma (St. Louis, MO, USA), Biogel was supplied by Bio-Rad (Rochester, CA, USA) and sodium metavanadate (NaVO_3) by Aldrich-Chemie (Steinheim, Germany). The $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was prepared by Dr M. Havranek from The Institute of Nuclear Biology and Radiochemistry, Czechoslovak Academy of Sciences, Prague. All other reagents were of grade purity (Lachema, Brno, CS).

Citrate lyase (CL) was prepared and purified according to the published procedure [15] based on polyethyleneglycol and ammonium sulfate fractionation followed by DEAE-cellulose and Biogel chromatography.

The activity of CL was assayed by measuring formation of ox-

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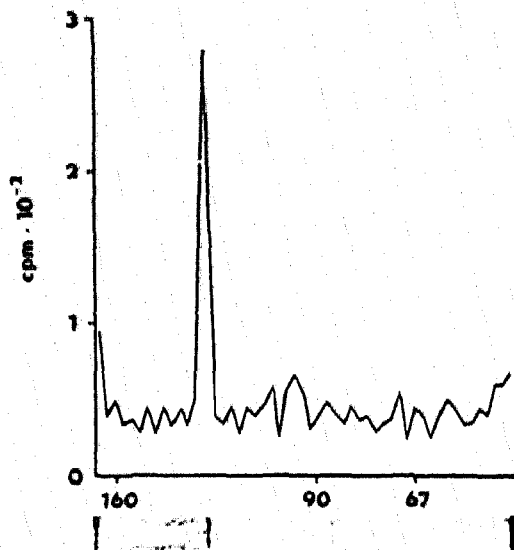


Fig. 1. SDS-polyacrylamide slab gel electrophoresis of the purified ATP-citrate lyase. Upper part of the figure shows the distribution of the ^{32}P radioactivity along the strip of the dried gel dissected into 2 mm bands. Numbers at the bottom represent M_r of the protein standards in kDa. For more details see section 2.

aloacetic acid by the method of Hestrin based on colorimetric determination of the complex formed by hydroxamic acids with FeCl_3 [16].

For endogenous protein phosphorylation the enzyme preparation was incubated in a medium (final volume 50 μl) consisting of 30 mM Tris-HCl, pH 7.4, 10 mM MgCl_2 , 0.4 mM EGTA, the enzyme preparation, 0.02 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (approx. 4000 cpm per pmol), and vanadate as indicated. The reaction was started by addition of ATP and after 20 s terminated by a conventional sample solution [17] or by the acidic 'stop' solution [18] to cleave the acid labile P-N bond of the histidine phosphate.

The system of Laemmli [17] was used for SDS polyacrylamide slab gel electrophoresis. Four per cent and 8% polyacrylamide was used for stacking and separation gels, respectively. The gels were stained and destained by a published procedure [19]. For quantitative measurements of radioactivity, the dried gels were dissected into 2 mm bands which were transferred into vials containing a scintillation cocktail based on toluene and the radioactivity was measured by scintillation spectroscopy.

3. RESULTS

As can be seen on Fig. 1 a single band of M_r approx. 125 kDa was obtained after purification of CL. This is in good agreement with the published results [12-14]. Any phosphate radioactivity was confined to this band. Vanadate at 1 mM concentration significantly inhibited phosphorylation of CL when a conventional sample solution had been used for termination of endogenous phosphorylation preceding application of the sample onto the gel. When, however, histidine phosphate had been removed by acid hydrolysis no inhibition by vanadate was apparent (Fig. 2). Since the radioactivity of the band resistant to the effect of vanadate (Fig. 2, N) was comparable to that after acid hydrolysis, it is obvious that vanadate did not affect phosphorylation

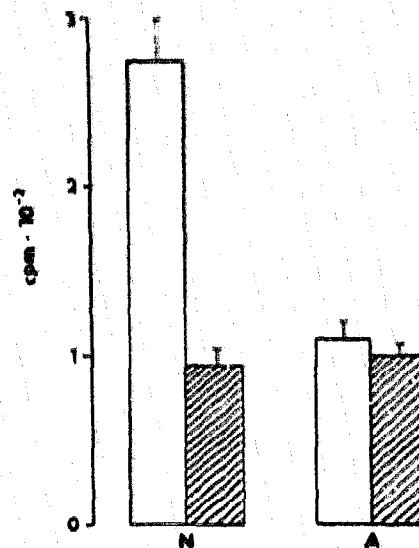


Fig. 2. Effect of vanadate on the phosphorylation of citrate lyase. Endogenous phosphorylation was stopped either by normal sample solution (N) or by acidic stop solution (A). White columns: radioactivity in the absence of added vanadate; hatched columns: radioactivity in the presence of 1 mM vanadate. Results are expressed as mean of at least 4 measurements ($\pm$ SEM).

of either serine or threonine. Therefore, it is reasonable to assume that the histidine residue of CL accounts for vanadate inhibition.

Subtracting vanadate resistant radioactivity from the total radioactivity obtained with various vanadate concentrations, made it possible to estimate the concentration dependency of the inhibitory effect of vanadate. The inhibition is concentration dependent (Fig. 3). It can be shown that the dose-dependency curve is closely

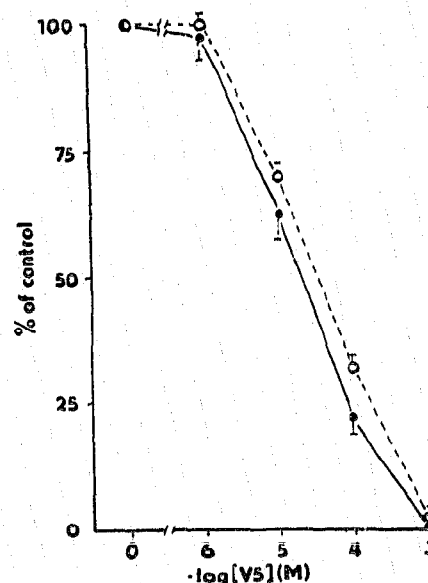


Fig. 3. Dose-dependence of the vanadate inhibition of citrate lyase ($\bullet$ --- $\bullet$) as compared to that of succinyl-CoA synthetase ($\circ$ --- $\circ$).

similar to that previously established for SCS (Fig. 3). Fifty per cent inhibition was achieved at vanadate concentrations 2×10^{-5} M and $3 \times 5 \times 10^{-5}$ M for CL and SCS, respectively.

4. DISCUSSION

The present results suggest that besides stimulation of phosphorylation of protein-bound tyrosine, inhibition of phosphorylation of the histidine residue in proteins may represent another general effect of vanadate. This may also be supported by a quantitative similarity of the vanadate effect on CL phosphorylation with that on the phosphorylation of SCS containing histidine as a single phosphorylatable amino acid [10,11].

In the present experiments we have not separated P-histidine from the CL hydrolysates, so that the presented evidence is rather indirect. However, taking into account that the structure of CL is established [12-14] and that the only acid labile P-amino acid in it is histidine, it is most likely that the observed effect of vanadate does concern histidine phosphorylation. Phosphohistidine has also been identified in other enzymes catalyzing phosphate cleavage and transfer [20]. It is likely, therefore, that effect and mode of action of vanadate will be identical for this group of enzymes. The detailed mechanisms of the vanadate effects on biochemical processes including protein phosphorylation, are not exactly known. Structural analogy with inorganic phosphate suggest at least for some enzymes formation of an intermediate that is much more stable than that formed by inorganic phosphate [6,21].

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