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INHIBITION OF PHOSPHORYLATION OF THE MITOCHONDRIAL 34 kDa PROTEIN

A UNIQUE EFFECT OF VANADIUM IONS?

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Abstract—We have shown previously that vanadate stimulates overall endogenous phosphorylation of proteins in subcellular particulate fractions. In brain mitochondria there is a single peptide band of M_r (approx.) 34 kDa, phosphorylation of which is inhibited rather than stimulated by both vanadate and vanadyl ions. Here, further characterization of this unique effect of vanadium ions is reported. Phosphorylation of the 34 kDa protein proceeds in the Triton X-100 extracts of mitochondria. The P-labeled 34 kDa band was recovered from TCA sediments of endogenously phosphorylated mitochondria. Acid lability of the phosphate linkage suggests a bond of P–N type. Phosphorylation of the 34 kDa protein is highly sensitive to Mg^{2+}, while Mn^{2+} is a less potent activator. The results provide further evidence for existence of a protein occurring exclusively in mitochondria, the phosphorylation of which is selectively modified by both vanadate anion and vanadyl cation in a way differing from those hitherto described.

Vanadium ions, particularly pentavalent anion-vanadate, (VO^{3-}, H_2VO^{4-}) and tetravalent cation-vanadyl (VO^{2+}) produce a wide range of biochemical effects [1, 2]. Their possible involvement in regulation of protein phosphorylation was suggested by demonstration that vanadate and, in several cases also, vanadyl, stimulate cAMP-dependent protein kinase [3–5], numerous tyrosine kinases [6–9] and inhibit many types of tyrosine phosphatases [7–10]. Vanadate has been shown to stimulate endogenous phosphorylation of the phospho-tyrosine proteins like the 95 kDa subunit of the insulin receptor [6–18], the 55 kDa and 61 kDa proteins from Raji human lymphoblastoma cells membrane [9], the 170 kDa protein of A-341 cell membrane [10] and the 32 kDa protein of Rous sarcoma virus-transformed chicken embryo fibroblasts [7]. Also, enhancement of endogenous phosphorylation of various brain proteins has been demonstrated [5, 19, 20]. Thus only the stimulatory effect of vanadate has been reported up to now. It may proceed by stimulation of appropriate kinases or by inhibition of corresponding phosphatases. However, recently we have been able to demonstrate in brain a peptide band, occurring in mitochondria, the phosphorylation of which was inhibited by both vanadate and vanadyl [20]. The aim of the present communication is to further characterize this protein and its phosphorylation.

MATERIALS AND METHODS

Animals. The cerebral cortex of male hooded rats (Long–Evans strain) weighing about 200 g were the source of mitochondria.

Chemicals. Vanadate was either from Fisher Co., (München, F.R.G.). Vanadyl sulfate was supplied by Jansen Chimica (Beerse, Belgium). [γ-^{32}P]ATP (s.a. 111 TBq per mmol) was obtained from Radiochemical Centre (Amersham, U.K.), [γ-^{33}P]ATP (s.a. about 8.5 TBq per mmol) was prepared by Dr M. Havranek from The Institute of Nuclear Biology and Radiochemistry, Czechoslovak Academy of Sciences. All reagents for polyacrylamide gel electrophoresis were from Serva (Heidelberg, F.R.G.). Triton X-100 and 2-mercaptoethanol were from Sigma Chemical Co. (St Louis, MO).

Preparation of mitochondria. A flotation-density gradient centrifugation of the lysed mitochondria [21] was used for preparation of perikaryal + synaptosomal mitochondria from the cerebral cortex. The resulting pellet was washed with 4 mM imidazol buffer, pH 7.4 and stored at −20° in a mixture of imidazol buffer–glycerol 1:1 (v/v).

Endogenous phosphorylation assay. The incubation medium of a total volume 50 μl consisted of (in mM) Tris–HCl buffer, pH 7.4 (50); $MgCl_2$, (10); EGTA, (2); DTT, (1); [γ-^{32}P]ATP, (0.02; 2000 cpm per pmol) or [γ-^{33}P]ATP, (0.02; 4000 cpm per pmol); about 100 μg mitochondrial proteins. After 60 sec preincubation with labeled ATP, phosphorylation was started by adding mitochondrial suspension followed by 15 sec incubation at 30°. The reaction was stopped by adding 50 μl of Laemmli's [22] stop solution and boiling for 3 min or as indicated in the legends to the figures or in text.

One-dimensional polyacrylamide gel electrophoresis. Slab gel electrophoresis of Laemmli [22] was used without modification. In the most cases 4 and 10% acrylamide gels were used for stacking and separation gels respectively. Usually, 60 μg of mitochondrial proteins were loaded on to the gel. After staining, destaining [23] and drying the gels were exposed to FOMA Medic Rapid X-Ray film.

Quantification of the radioactivity of the individual

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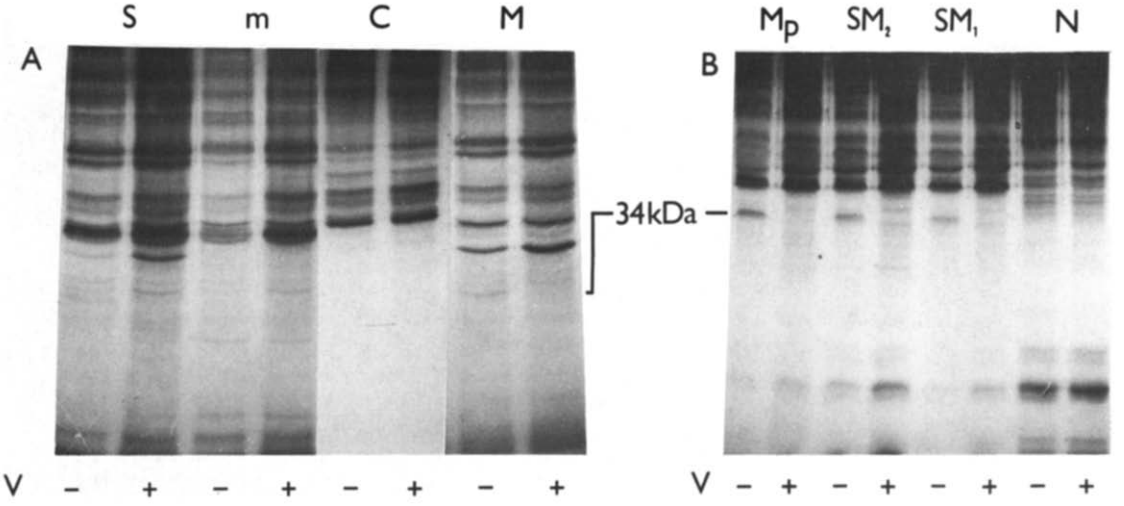

Fig. 1. Effect of vanadate (V, 10^{-3} M) on endogenous phosphorylation of proteins from subcellular particles of the rat cerebral cortex. (A) Autoradiograms of the phosphorylated protein bands separated in 10% polyacrylamide gels: synaptosomes (S); microsomes (m); cytosol (C); mitochondria (M). (B) Autoradiograms of the phosphorylated protein bands separated in 12% gels: perikaryal mitochondria (Mp); heavy (SM_2) and light (SM_1) synaptosomal mitochondria, and nuclei (N). See [20] for more detail in part (B). Subcellular particles in part (A) were obtained by standard procedure [37].

bands was achieved by dissection of the appropriate band from the stained electrophoreograms. The bands were transferred into the scintillation cocktail based on toluene and radioactivity was determined by means of liquid scintillation. For the blank determination the sections of gels revealing no detectable radioactivity on autoradiogram (R_f approx. 0.76) were counted. In some cases, densitometry scanning of the autoradiograms by Shimadzu Double-beam TLC scanner was performed.

Solubilization of mitochondrial proteins. Mitochondria suspended in a buffer solution consisting of 6 mM Tris–HCl, pH 8.1 and 0.1 mM DTT were mixed with the same volume of 1% Triton X-100 in the same buffer. After vigorous shaking the mixture was kept on ice under intermittent mixing [24]. After spinning for 30 min at 10,000 *g* both the mitochondrial pellet resuspended in Tris–HCl buffer, pH 7.4 and a portion of the supernatant fluid (after dialysis) were subjected to endogenous phosphorylation assay as described above.

Testing the acid-lability of the phosphate bond in the 34 kDa protein. Endogenous phosphorylation of the mitochondrial suspension was stopped by "acidic stop solution" [25] consisting of 10.5% sodium dodecyl sulfate, 10.5% 2-mercaptoethanol, 1 M HCl, 3.5 mM EDTA and 17.5% glycerol. The mixture was kept at room temperature for 30 min and neutralized with Tris using Bromphenol Red as an indicator. Samples were then analyzed by polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Figure 1 demonstrates the effect of vanadate on endogenous phosphorylation of proteins from synaptosomes, microsomes, cytosol, mitochondrial and nuclear fractions of the cerebral cortex of the rat. Phosphorylation of overall proteins is stimulated by vanadate. It has been shown that this stimulation is not due exclusively to inhibition by vanadate of ATPase activity [5, 20]. However, there is a single band of M_r approx. 34 kDa occurring only in mitochondria (total, light and heavy synaptosomal mitochondria) phosphorylation of which is markedly inhibited by vanadate. The presence of synaptosomal mitochondria explains availability of 34 kDa protein and inhibition of its phosphorylation by vanadate in the synaptosomal fraction. No 34 kDa phosphorylation and its inhibition by vanadate is observable in synaptic membranes [20], microsomes, cytosol or nuclei (Fig. 1). The same effect has been observed in mitochondria from rat liver and brown adipose tissue from golden hamster as well as from bovine heart muscle (not shown).

Vanadyl cation has qualitatively the same effect, but its potency is by about one order of magnitude lower than that of vanadate. This is shown in Fig. 2, summarizing the concentration dependence of the inhibitory action of both vanadate and vanadyl ions which was described earlier in more detail [20].

Characterization of the phosphate bond in the 34 kDa protein

The fact that the 34 kDa could be recovered from the TCA sediments suggests a macromolecular nature of the phosphorylated substance (not shown). Phosphorylation of the mitochondrial 34 kDa protein proceeds in the Triton X-100 extracts but not in Triton X-100 sediments. It seems likely, therefore, that the system controlling phosphorylation of the 34 kDa protein, as well as the 34 kDa protein itself, are loosely bound to some of the mitochondrial membranes (see Fig. 3).

The amino acid in the 34 kDa protein to which phosphate is bound has not yet been established.

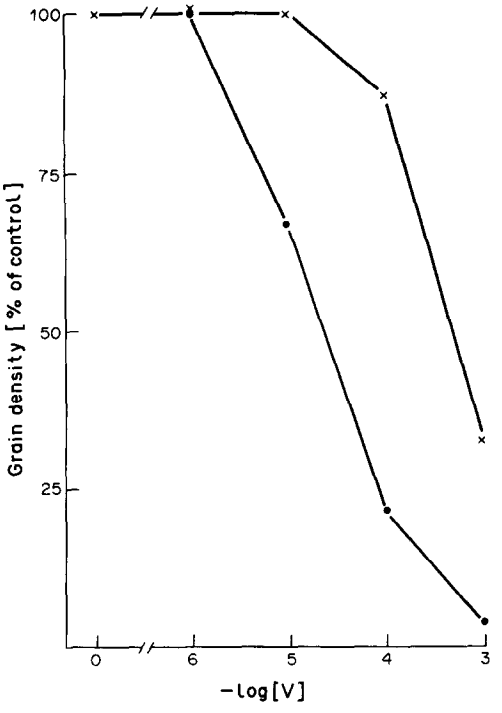

Fig. 2. Effect of vanadate (● — ●) and vanadyl (× — ×) on endogenous phosphorylation of the 34 kDa protein of cerebral cortex mitochondria. Effect of vanadyl was tested in the presence of 1 mM dithiothreitol to protect vanadyl from oxidation. Quantitation was performed by scanning the autoradiograms. The values represent means of at least three measurements.

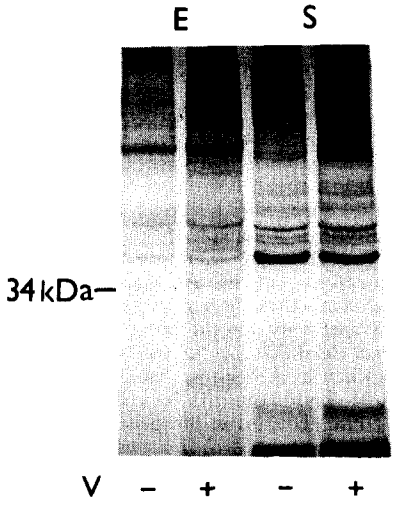

Fig. 3. Endogenous phosphorylation of the cerebral cortex mitochondria pretreated with 0.5% (v/v) Triton X-100. (E), phosphorylation in the fraction of mitochondria extracted by Triton. (S), phosphorylation in the Triton sediments. V, 10^{-3} M vanadate. For details see Materials and Methods.

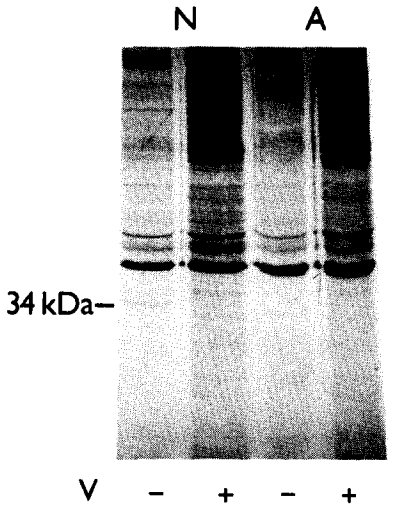

Fig. 4. Phosphate labelling of the mitochondrial proteins after quenching of the endogenous phosphorylation with the Laemmli's stop solution (N) or with stop solution containing 1 M HCl (A). V, 10^{-3} M vanadate. See Materials and Methods.

However, the nature of the phosphate bond can be estimated from the following data. First, the presence of tyrosine phosphate was excluded [20]. Second, resistance of P label in the 34 kDa protein to hydroxylamine treatment eliminates also an acyl–phosphate bond (not shown). Finally, removal of the phosphate label from the 34 kDa protein by quenching endogenous protein phosphorylation reaction by an "acidic stop solution" (Fig. 4) suggests the acid-labile nature of the P bond. It is most likely, therefore, that this is a P–N bond which is susceptible to vanadium. Thus, probable candidates for the critical amino acid could be limited to lysine, arginine and histidine.

Characterization of the 34 kDa phosphorylation

It has been shown previously that phosphorylation of the 34 kDa protein is influenced by neither of the following treatments: addition of cAMP, Ca^{2+}, Ca–calmodulin complex, Ca + phosphatidyl serine + protein kinase C, shift of pH from 6.6 to 8.1 [20]. Here we show that phosphorylation of this protein is highly sensitive to Mg^{2+}. A decrease of Mg concentration by an order of magnitude (from 10 mM to 1 mM) had almost no effect (90% control) on the extent of the 34 kDa labeling. Even in media devoid of added Mg^{2+}, phosphorylation of the 34 kDa declined to 57% of control. Mn^{2+} is less effective than Mg^{2+} (Fig. 5). According to our unpublished results the inhibition by vanadium ions might at least partially be due to stimulation of a tentative 34 kDa protein phosphatase.

DISCUSSION

All hitherto published results show that vanadate induces enhancement of protein phosphorylation.

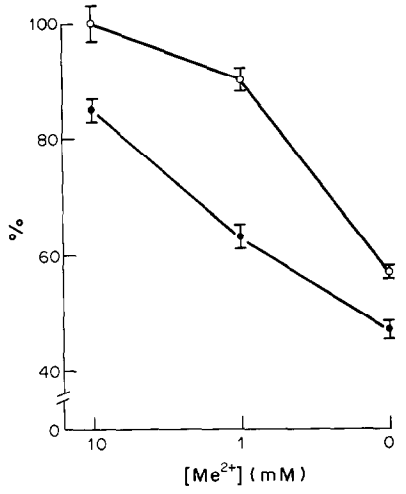

Fig. 5. Effect of Mn^{2+} (● — ●) on endogenous phosphorylation of the 34 kDa protein of cerebral cortex mitochondria as compared to that of Mg^{2+} (○ — ○). Quantitative evaluation was performed by liquid scintillation measurement of radioactivity of the bands dissected out of the dried electrophoreograms. The values represent means ± SE of four measurements.

The present study characterizes in more detail inhibition of phosphorylation of a single protein occurring exclusively in mitochondria of mammals.

The unexpected results might be due to some artifact but the following observations make it unlikely. The 34 kDa protein is recovered from the TCA sediments of mitochondrial suspensions, suggesting its macromolecular nature. It is also recovered from the Triton X-100 treated mitochondria. It occurs exclusively in mitochondria regardless of their origin and method of preparation. On the other hand, non-mitochondrial particles obtained from the same tissue by the same general procedure and subjected to the same storage, endogenous phosphorylation, as well as protein separation schedules, did not contain any peptide band, phosphorylation of which is inhibited by vanadium ions.

The exact nature of the 34 kDa protein has not yet been elucidated and the type of bond linking phosphate to the amino acid(s) is not known. Our experiments provide some indirect evidence that this is an acid-labile P–N type bond by which phosphate is bound to some basic amino acid (lysine, arginine, histidine) of the 34 kDa protein. This characteristic reminds one of the alfa-subunit of succinic thiokinase (succinyl-CoA synthetase). In our preliminary experiments vanadate appeared to inhibit phosphorylation of the alfa-subunit of a commercial succinic thiokinase.

The possible mode of action of vanadium ions on protein phosphorylation is not fully understood. Only the mechanisms of their effect on tyrosine–protein phosphorylation has been hypothesized [26, 27].

It has been suggested that tetravalent vanadium cation (vanadyl) is the physiologically active form of this element [28]. In a variety of systems, including that used in the present study, vanadyl has been shown to be less effective than vanadate [29–33]. This quantitative variability may reflect differences in electrical charge and redox state, as well as in accessibility to the site of their action [31].

No unambiguous evidence has been provided as yet for the regulatory role of vanadium ions in living organisms. The only exception is a special type of azotobacter nitrogenase [34, 35] and perhaps bromoperoxidase from marine alga *Ascophyllum nodosum* [36] in which vanadium forms a part of their prostetic groups.

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