FEBS Letters 376 (1995) 58–60 FEBS 16316

Binding of vanadium (IV) to the phosphatase calcineurin

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Received 13 July 1995; revised version received 12 October 1995

Abstract X-band electron spin resonance spectroscopy was used to study the binding of vanadium (IV), or vanadyl, to the brain serine/threonine phosphatase-2B, calcineurin. Spectra were determined on frozen solutions of vanadyl and calcineurin at pH 7.4 in the presence of 20% (v/v) glycerol. The binding of vanadyl to the enzyme was established, and the data suggested the presence of two classes of sites, the higher affinity class of which contained two binding sites for vanadyl. The calcium-binding B subunit of the heterodimeric protein was also shown to bind vanadyl. The holoprotein appeared to be stabilized by vanadyl, and vanadyl enhanced enzymatic activity when assayed with or without calmodulin in the absence of calcium.

Key words: Calcineurin; Phosphatase; Electron Spin Resonance; Vanadium (IV); Vanadyl

1. Introduction

The important serine/threonine protein phosphatase-2B, calcineurin, exists as a heterodimer and requires metal ions and calmodulin for full enzymatic activity [1]. The A subunit (M_r 61 kDa) contains the catalytic site and binding region for calmodulin; the B subunit (M_r 19 kDa) contains four calcium-binding sites in the form of 'EF hands' and exhibits homology with calmodulin and troponin C [1]. Genomic and complementary DNA sequences are available for the A and B subunits from several species and indicate a high degree of conservation of structure during evolution [2–4].

Of considerable interest were the findings that two structurally unrelated immunosuppressants that inhibit T lymphocyte activation and lymphokine production, cyclosporin A and FK506, both of which are derived from mold fermentation broths and bind respectively to cyclophilin and FK binding protein, i.e. immunophilins, inhibit calcineurin activity [5,6]. Recent studies have shown that a target protein of calcineurin is the nuclear factor of activated T cells, NF-AT, a phosphorylated transcriptional activator of the interleukin-2 gene [7,8].

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Vanadium, an essential trace element in experimental animals [9], mimics many of the actions of insulin [10] and may have clinical potential for the treatment of diabetes and insulin resistance [11]. It is found in vivo primarily in its V oxidation state, vanadate; in turn, this is reduced intracellulary to the IV oxidation state, vanadyl [12]. ESR spectroscopy has been used to demonstrate that vanadyl binds to a variety of metalloproteins [13,14]. We have shown that vanadyl binds to calmodulin [15,16] and inhibits the stimulation of skeletal muscle myosin light chain kinase activity [17]. In view of the homology of the calcineurin B subunit and calmodulin, particularly in the regions of calcium-binding [1], we performed ESR spectroscopy to ascertain if vanadyl binds to brain calcineurin. Our results show that vanadyl binds to this protein phosphatase and appears to enhance enzymatic activity.

2. Experimental

2.1. Preparation of calcineurin/subunits and enzymatic assay

Calcineurin was purified to homogeneity from bovine brain by ammonium sulfate precipitation followed by column chromatography: DE-52 cellulose, Affi-gel-Blue, calmodulin-Sepharose 4B (affinity), and Sephacryl 200 [18,19]. Subunits were prepared by dialyzing calcineurin against 6 M urea for 4–6 h in a buffer containing 0.1 M HEPES, 5 mM dithiothreitol, 15 mM EDTA, and 80 mM LiBr, pH 7.4, followed by chromatography on a DEAE-Sephadex CL-6B column which resolved the two subunits. Metal ions were removed from the B subunit fraction, and this was concentrated with a Centricon 10 column (Amicon). Calcineurin activity was determined as described by Li [20] after dilution in 50 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 0.2 mg/ml bovine serum albumin. The assay solution contained 20 mM *p*-nitrophenyl phosphate, 0.1 mM $CaCl_2$, 0.5 mM $MnCl_2$, and 0.3 μM calmodulin.

2.2. ESR spectroscopy

X-band (9.2 GHz) ESR spectra were measured using an IBM-Bruker (ER-200 SRC) ESR spectrometer equipped with an IBM-9000 computer and a dual TE_{104} cavity. Repetitive scans, typically 5–7, were stored, and the data were subjected to standard signal averaging and baseline corrections. Spectra were obtained on liquid (298 K) and frozen (125 K) solutions. However, since calcineurin appeared unstable for long periods of time at 298 K and since the concentration of each sample was low, higher sensitivity was achieved by freezing the samples which yielded spectra near the rigid limit. Stock vanadyl solutions of about 0.1 M were prepared by dissolving vanadyl sulfate in deionized, doubly distilled water and adjusting the pH to 2 with 6 N HCl. The solutions were degassed, and exact concentrations were determined spectrophotometrically at 750 nm using an extinction coefficient of 18 $M^{-1} \cdot cm^{-1}$ [21]. Aliquots of the vanadyl stock solution were titrated into protein solutions (ca. 0.1 mM calcineurin in 0.15 ml of 0.1 M HEPES, pH 7.4, 0.1 M NaCl, and 0.5 mM dithiothreitol), which had been purged with N_2, under constant stirring. After 5 min of additional stirring, the solutions were transferred to quartz capillary tubes (3 mm o.d., Wilmad), sealed with paraffin tape, and cooled in a dry ice-acetone bath. The sample was then placed in one of the ESR cavities; the other cavity contained 1 mM $VOSO_4$ in 20% aqueous glycerol, pH 2, in a quartz flat cell as a concentration standard. The temperature was controlled at 125 K with a Bruker ER 4111 VT.

SSDI 0014-5793(95)01245-1

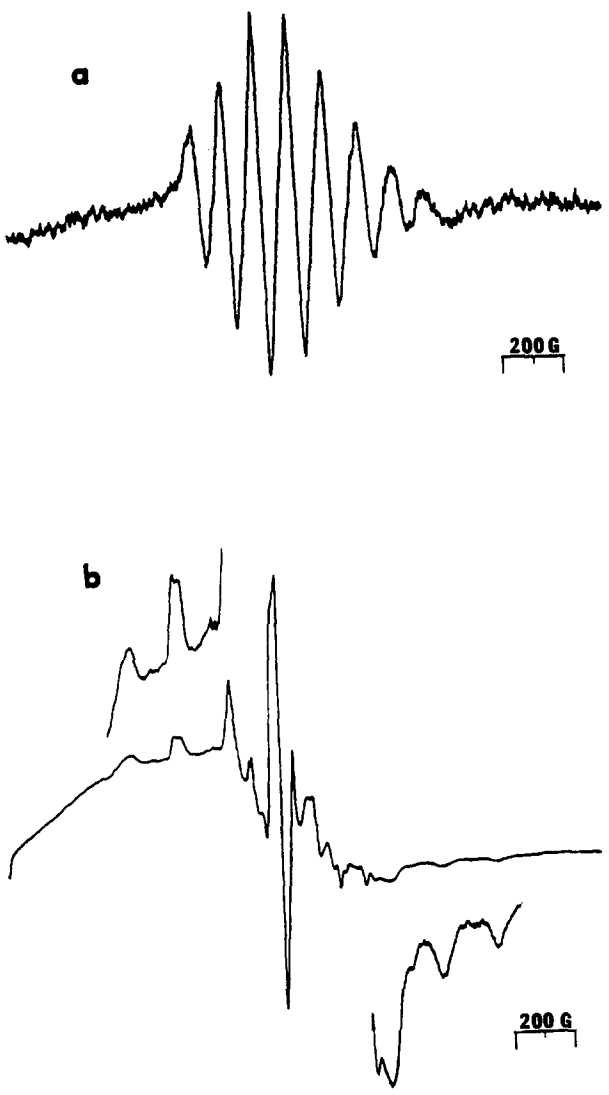

Fig. 1. ESR spectra of: (a) vanadyl at pH 2 and 125 K in the presence of 20% glycerol (v/v), and (b) of vanadyl/calcineurin (8:1 molar equivalents) at pH 7.4 and 125 K in the presence of 20% (v/v) glycerol (the high field and second low field resonance peaks are amplified).

3 Results and discussion

The ESR spectra of vanadyl at 125 K (pH 2) and of vanadyl/calcineurin (8:1 molar ratio) at 125 K (pH 7.4) are shown in Fig. 1a and 1b, respectively. Since vanadyl is ESR silent at pH 7.4, the spectrum obtained in the presence of protein is indicative of vanadyl binding to the phosphatase and differs considerably from that of free vanadyl. A splitting of the second low field resonance is apparent, and, in addition, the high field and center peaks are skewed (Fig. 1b). These findings are suggestive of at least two classes of binding sites.

The intensity of the vanadyl center peak was determined at various molar ratios of vanadyl:calcineurin under the same conditions (Fig. 2). Calcineurin is relatively unstable, and thus a fresh aliquot of protein was used for each vanadyl equivalent; the utilization of the dual cavity technique enabled us to obtain considerable relative accuracy. Assay of the enzyme following recording of the ESR spectra showed that 90% of the activity was recovered. The center peak intensity increased in an approximate linear fashion upon addition of the first two vanadyl equivalents, then there was no change in signal between the addition of the third and fifth equivalents, and finally a small increase in intensity was observed as additional vanadyl was added to a final vanadyl/enzyme ratio of 8:1. These results are consistent with the presence of two classes of vanadyl sites on calcineurin. When two equivalents of calcium were added to calcineurin, followed by the addition of two equivalents of vanadyl, the appearance of an ESR spectrum (not shown) indicated that calcium and vanadyl bind to distinct sites on the phosphatase or that vanadyl has a higher affinity than calcium for the first two metal binding sites.

Since only very limited amounts of purified subunits were available, we were unable to obtain definitive ESR spectra of vanadyl and the A and B subunits. A preliminary spectrum is shown in Fig. 3 for the B subunit with 6 equivalents of vanadyl (125 K, pH 7.4). The spectrum exhibits near 'rigid limit' properties and is similar to that of vanadyl with the holoprotein. Yet, differences do exist, probably attributable to binding of vanadyl to the A subunit or perhaps to subunit conformational changes associated with heterodimer formation that affects binding.

In view of the apparent stabilization of calcineurin by vanadyl, enzymatic activity was measured in the presence of vanadyl (Fig. 4). Of considerable interest was the finding that

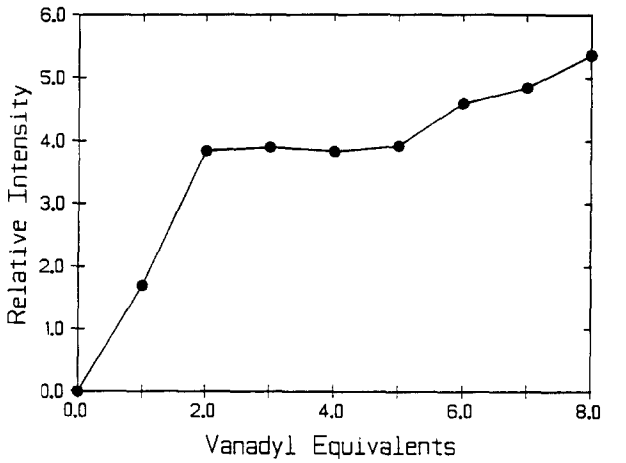

Fig. 2. Normalized center field peak intensity of vanadyl at various vanadyl/calcineurin molar ratios at pH 7.4, 125 K in the presence of 20% (v/v) glycerol. Each point represents a fresh aliquot of protein.

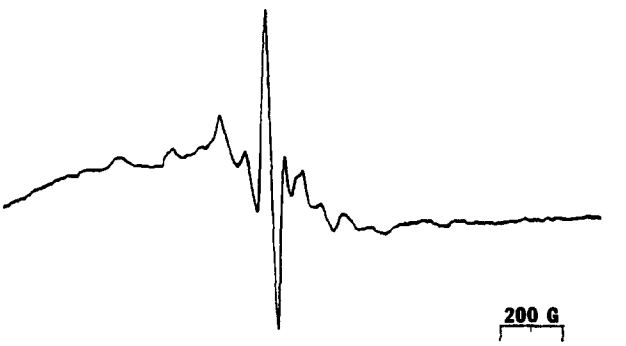

Fig. 3. ESR spectrum of vanadyl in the presence of the calcineurin B subunit at a molar ratio of 6:1 (pH 7.4, 125 K) in the presence of 20% (v/v) glycerol.

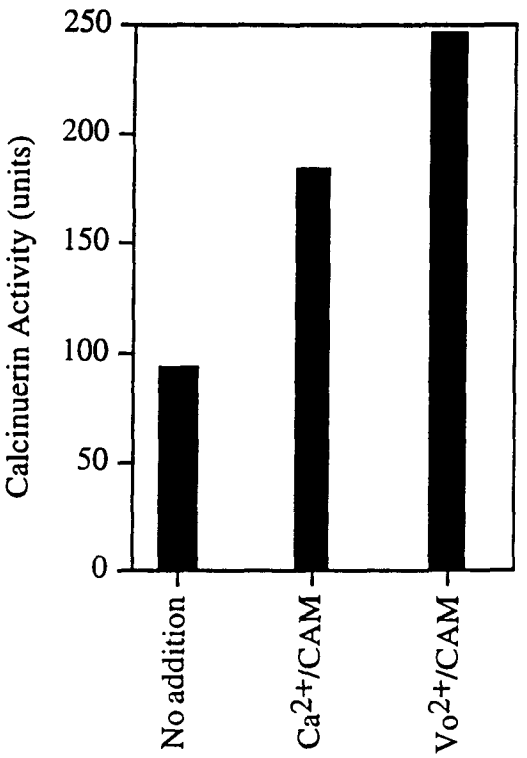

Fig. 4. Effect of vanadyl on calcineurin activity, which was assayed using *p*-nitrophenyl phosphate as a substrate. Calcium and vanadyl were present at 0.1 mM where added.

vanadyl leads to higher enzymatic activity than does calcium, a finding that was consistently observed in several independent assays under slightly varying conditions. Moreover, increased calcineurin activity was noted under comparable assay conditions, but in the absence of calcium and calmodulin (data not shown). Thus, vanadyl can interact directly with the A or B subunit, or both, and enhance enzymatic activity. We have shown that vanadyl also binds to calmodulin [15,16], although the vanadyl-calmodulin complex does not stimulate skeletal muscle myosin light chain kinase activity [17], as does calcium-calmodulin. A role of vanadyl-calmodulin in calcineurin regulation could not be directly determined since vanadyl is capable of directly enhancing enzymatic activity.

In summary, this study has demonstrated that vanadyl binds to calcineurin and can stimulate enzymatic activity in the absence of calcium and calmodulin.

Acknowledgements: This work was supported by NIH GM35415.

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