

Research article

## The Metal Coordination of sCD39 during ATP Hydrolysis

Wei Chen and Guido Guidotti\*

Address: Department of Molecular and Cellular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA

E-mail: Wei Chen - weichen@fas.harvard.edu; Guido Guidotti\* - guidotti@fas.harvard.edu

\*Corresponding author

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### Abstract

**Background:** The hydrolysis of ATP and ADP by ecto-nucleoside triphosphate diphosphohydrolase I (CD39) requires divalent cations, like  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . In spite of considerable work, it is not clear whether divalent cations bind to the enzyme in the absence of nucleotide or only as nucleotide- $\text{Me}^{+2}$  complex. Here we study the protein ligands for  $\text{Me}^{+2}$ .

**Results:** When  $\text{VO}^{2+}$  was used as a substitute for  $\text{Ca}^{2+}$ , the ATPase activity of soluble CD39 was 25% of that with  $\text{Ca}^{2+}$  as cofactor. Protein ligands of the  $\text{VO}^{2+}$ -nucleotide complex bound to the catalytic site of soluble CD39 were characterized by electron paramagnetic resonance (EPR) spectroscopy. The EPR spectrum contained one species designated T with  $\text{VO}^{2+}$ -AMPPNP as ligand. Two species D1 and D2 were observed when  $\text{VO}^{2+}$ -AMPCP was bound to soluble CD39. The results suggest that species D1 and D2 represent the metal-ADP complexes at the catalytic site of soluble CD39 corresponding to the intermediate formed during ATP hydrolysis and the substrate for further hydrolysis, respectively.

**Conclusions:**  $\text{VO}^{2+}$  can functionally substitute for  $\text{Ca}^{2+}$  as a cofactor of sCD39, and it produces four different EPR features when bound in the presence of different nucleotides or in the absence of nucleotide. The metal coordination for each conformation corresponding to each EPR species is proposed, and the mechanism of sCD39 catalysis is discussed.

### Background

Ecto-Nucleoside triphosphate diphosphohydrolases (E-NTPDases, formerly called ecto-ATPases) hydrolyze nucleotides in the presence of divalent cations and are insensitive to inhibitors of P-type, F-type, and V-type ATPases [1]. Three isoforms that differ in the ratio of ATPase/ADPase activity are present on the cell surface [2]: E-NTPDase1 with a ratio of 1, E-NTPDase2 with a ratio of 10 and E-NTPDase3 with a ratio of 3–5. NTPDases are important in many physiological processes like cell motility, adhesion, nonsynaptic information transfer, secretion, regulation of hemostasis and ectokinases [1]. Understanding the enzymatic mechanisms of the NTP-

Dases will help description of their physiological functions, and development of strategies to regulate the functions of the enzymes.

The catalytic mechanism of NTPDases is not known even though some basic facts of the catalysis have been established. NTPDases do not form phosphorylated intermediates during catalysis, a conclusion also supported by lack of vanadate sensitivity and  $\text{P}_i$  product inhibition [3–6]. The catalytic reaction appears to be irreversible and no partial reactions have been observed [7,8]. Divalent cations like  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  are required for activity, and maximal activity is reached when the concentrations of

substrates and divalent cations are equal [1]. The specific activities of NTPDases vary over a broad range from ten thousand units for potato apyrase to less than one hundred units for chicken gizzard ecto-ATPase [9,10]. Sequence comparisons indicate that most of NTPDases contain five highly conserved regions, apyrase conserved region, ACR1 – ACR5 [9,11]. However, the catalytic sites have not been identified, although ACR1 and ACR4 have been implicated in  $\beta$ - and  $\gamma$ -phosphate binding, respectively [9].

E-NTPDase1 is also called CD39, as it was first described as an antigen present on activated B and T lymphocytes. Residues of ACR1 to ACR5 of CD39 have been mutated to study the involvement of the ACR regions in catalysis. E174 in ACR3 and S218 in ACR4 are required for catalytic function [12]. Substitution of H59 in ACR1 converted CD39 into an ADPase in a quaternary structure dependent manner [13]. Mutation of W187A in ACR3 affected CD39 folding and translocation, while mutation of W459A in ACR5 increased ATPase activity but diminished ADPase activity [14]. Mutations of D62 and G64 of ACR1 and D219 and G221 of ACR4 demonstrated that the nucleotide phosphate binding domains of NTPDases are similar to those present in the actin/heat shock protein/sugar kinase superfamily [15]. These results suggest that the conserved residues of the ACR1 to 5 regions are involved in the catalytic mechanism of CD39.

The catalytic activity of CD39 is dependent on the presence of divalent cations. Since the interactions of  $\text{Ca}^{2+}$  and  $\text{Mg}^{+2}$  with proteins are difficult to study due to the lack of spectroscopic properties, vanadyl ( $\text{V}^{\text{IV}}=\text{O}$ ) $^{2+}$  has been used as a probe of the ligands that compose  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Mn}^{2+}$  binding sites of several proteins, including carboxypeptidase [16], S-adenosylmethionine synthetase [17,18], pyruvate kinase [19,20], and  $\text{F}_1$ -ATPase [21,22]. This cation specifically binds to divalent cation binding sites of several enzymes, and in many cases serves as a functional cofactor [23]. Vanadyl has one axial and four equatorial coordination sites relative to the axis of the double-bonded oxygen, an arrangement that is similar to that for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . As it is known that the **A** and **g** tensors derived from the EPR spectrum of bound  $\text{VO}^{2+}$  are a direct measure of the nature of the equatorial metal ligands [24], binding of  $\text{VO}^{2+}$  to CD39 could provide details about the catalytic mechanism of CD39.

Recently we reported that a recombinant soluble CD39, capable of hydrolyzing both ATP and ADP, was expressed and purified from insect cells [25]. Only one nucleotide-binding site was identified on the purified soluble CD39 in the presence of  $\text{Ca}^{2+}$  when non-hydrolysable nucleotide analogs were used. In this report, we

characterized the signals that were obtained from bound  $\text{VO}^{2+}$  when ATP or ADP was present at the catalytic site of the purified soluble CD39. The possible metal ligands for  $\text{VO}^{2+}$  at the catalytic site are proposed and the catalytic mechanism is discussed.

## Results

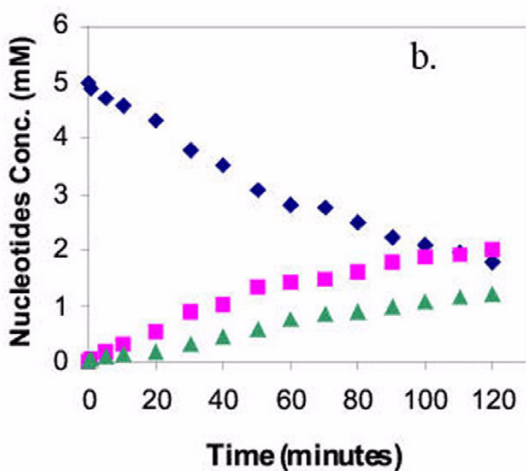
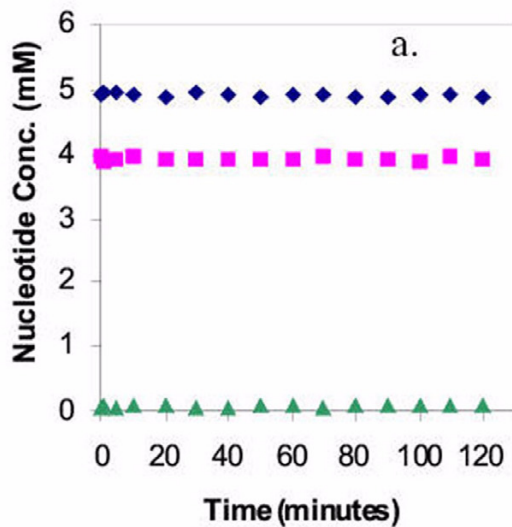
### **Nucleotidase activity of purified soluble CD39 with $\text{VO}^{2+}$ as cofactor**

The ability of purified soluble CD39 to hydrolyze  $\text{VO}^{2+}$ ATP is shown in Figure 1. Soluble CD39 did not hydrolyze either ATP or ADP in the absence of  $\text{VO}^{2+}$  (Fig. 1A). When  $\text{VO}^{2+}$  was mixed with ATP at a ratio of 1:1, the concentrations of both ADP and AMP increased and ATP decreased as the incubation time was prolonged (Fig. 1B). The ATPase activity of sCD39 with  $\text{VO}^{2+}$  was about 25% of that with  $\text{Ca}^{2+}$  as a cofactor. Vanadyl is unstable in aqueous solution at pH7.0 in the absence of chelator and will precipitate out of solution as  $[\text{VO}(\text{OH})_2]_n$ . The rate of precipitation depends on the abundance and affinity of the chelator. This means that the actual  $\text{VO}^{2+}$  concentration was lower than 0.5 mM. This result indicates that  $\text{VO}^{2+}$  can functionally substitute for  $\text{Ca}^{2+}$  as cofactor for sCD39 nucleotidase activity.

### **Characterization of bound $\text{VO}^{2+}$ ADPNP by CW-EPR**

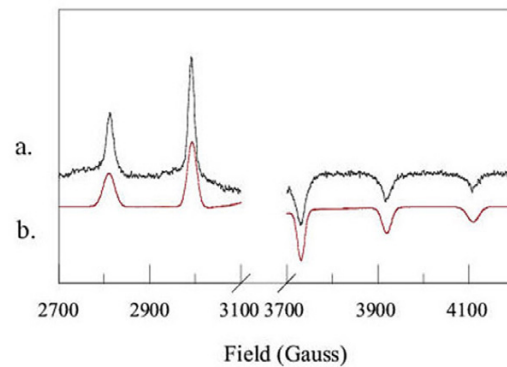
The parallel features of CW-EPR spectrum of bound  $\text{VO}^{2+}$  in the presence of ADPNP, an ATP analog, are shown in Figure 2a. This spectrum shows  $^{51}\text{V}$  hyperfine splitting and the center of the parallel transitions from molecules with the  $\text{V}=\text{O}$  bond oriented along the magnetic field ( $A_{||}$ ,  $g_{||}$ ) which are strong enough to tell the nature of  $\text{VO}^{2+}$  equatorial ligands [22]. Of the eight transitions that result from the parallel oriented molecules, the  $-7/2_{||}$ ,  $-5/2_{||}$ ,  $+3/2_{||}$ ,  $+5/2_{||}$ , and  $+7/2_{||}$  transitions (shown in the figures from left to right, respectively) do not overlap with perpendicular transitions. The  $^{51}\text{V}$  hyperfine splitting spectra from molecules with  $\text{V}=\text{O}$  bond perpendicular to the magnetic field ( $A_{\perp}$ ) are much smaller and not shown here [21]. The intensity of  $-5/2_{||}$  peak is used as direct measurement of the amount of bound  $\text{VO}^{2+}$ , since this peak is the most intense peak in the EPR spectrum that contains contribution only from  $A_{||}$  but not  $A_{\perp}$  [21,26]. In this study, the intensities of each bound  $\text{VO}^{2+}$ -EPR feature were normalized to 1 mg of protein.

$\text{VO}^{2+}$  bound as the  $\text{VO}^{2+}$ -AMPPNP complex to sCD39 produced a strong spectrum characterized by  $A_{||}$  of 504.25 MHz and  $g_{||}$  of 1.9410 (Fig. 2b), called species T (Table 1). The best fit of EPR species T to eq 1 is one equatorial nitrogen from an amino group and three equatorial oxygen ligands from carboxyl or phosphate groups (Table 2). This result is consistent with AMPPNP binding



**Figure 1**  
ATP hydrolysis by sCD39 with  $\text{VO}^{2+}$  as cofactor analyzed by HPLC. 2  $\mu\text{g}$  of sCD39 was included in each assay. A. The reaction was conducted in the presence of 0.5 mM ATP but without  $\text{VO}^{2+}$ . B. The reaction was initiated by adding 0.5 mM  $\text{VO}^{2+}$ -ATP (1:1). ATP is represented as black diamonds; ADP as a black square; and AMP as a black triangles;

strongly to a single site on sCD39 in the presence of metal [25].



**Figure 2**  
Parallel regions of  $\text{VO}^{2+}$ -EPR spectrum of  $\text{VO}^{2+}$ -ADPNP bound to sCD39. a. One mole equivalent of  $\text{VO}^{2+}$ -ADPNP was added to 2.8 mg of sCD39 as described in Experimental Procedures. EPR conditions were as follows: field modulation frequency, 100 kHz; modulation amplitude, 0.97 G; receive gain,  $2 \times 10^4$ ; sweep time 5.243 s; time constant 1.28 ms; micropower, 1.0 mW; microwave frequency 9.40386 GHz, 1000 scans; temperature; 125 K. b. Simulated spectrum was generated by the program QPOWA with the experimental conditions above and  $A_{||} = 504.25$  MHz,  $g_{||} = 1.9410$  for Species T.

#### Characterization of EPR species from $\text{VO}^{2+}$ -AMPCP bound to sCD39

Figure 3a shows the parallel features of the EPR spectrum of sCD39 bound  $\text{VO}^{2+}$ -AMPCP. Two sets of parallel transitions were observed, and the derived  $A_{||}$  and  $g_{||}$  values are listed in Table 1. One set had  $A_{||}$  of 521.78 MHz and  $g_{||}$  of 1.937, which is defined as species D1 (Fig. 3b). The other set displayed  $A_{||}$  of 490.01 MHz and  $g_{||}$  of 1.9435, which is called species D2 (Fig. 3c). The intensity of species D1 accounted for 11.4% of species T from bound  $\text{VO}^{2+}$ -AMPPNP, and the intensity of D2 accounted for 7.1% of species T. The intensity ratio of species D1 over D2 was 1.6.

In order to distinguish species D1 from D2, the sample with  $\text{VO}^{2+}$ -AMPCP bound to sCD39 was thawed and incubated at room temperature for 30 minutes, and the  $\text{VO}^{2+}$  EPR spectrum was collected again. As shown in Figure 4a and 4b, either  $A_{||}$  or  $g_{||}$  values for both species D1 and D2 were changed. The intensity of species D1 was not changed as it accounted for 12.1% of the intensity of species T of the bound  $\text{VO}^{2+}$ -AMPPNP. However, the intensity of species D2 was decreased dramatically, and it accounted for only 0.1% of the intensity of species T. The intensity ratio of D1 over D2 increased about 75 fold to become 120.

**Table 1: Experimental signal intensity and  $^{51}\text{V}$ -hyperfine parameters derived from the  $\text{VO}^{2+}$  bound to sCD39 under different conditions.**

Nucleotide	Species	Intensity	Experimental Parameters	
		(% of VOADPNP)	$g_{  }$	$A_{  }$
VOADPNP	T	100	1.9410	504.25
$\text{VO}^{2+}$	V	20.3	1.9460	486.00
	D1	11.4	1.9370	521.78
VOAMPCP	D2	7.1	1.9435	490.01
VOAMPCP*	D1	12.1	1.9350	521.78
VOATP	D2	7.5	1.9455	489.5
VOATP*	D2	0.2	1.9455	489.5

\* Samples were incubated at room temperature for 30 minutes.

**Table 2: Best fits of the  $^{51}\text{V}$ -hyperfine parameters (Eq. 1) of  $\text{VO}^{2+}$  bound to various equatorial ligands of sCD39 under different conditions.**

Species	Calculated		Most Probable Equatorial Ligands			
	$g_{  }$	$A_{  }$				
T	1.9445	504.25	$\text{RNH}_2$	$\text{RCOO}$	Pi	Pi
D1	1.9390	520.95	$\text{H}_2\text{O}$	$\text{RCOO}$	Pi	Pi
D2	1.9435	490.01	ROH	$\text{RCOO}$	$\text{RCOO}$	Pi
V	1.9500	485.36	ROH	ROH	$\text{H}_2\text{O}$	$\text{H}_2\text{O}$

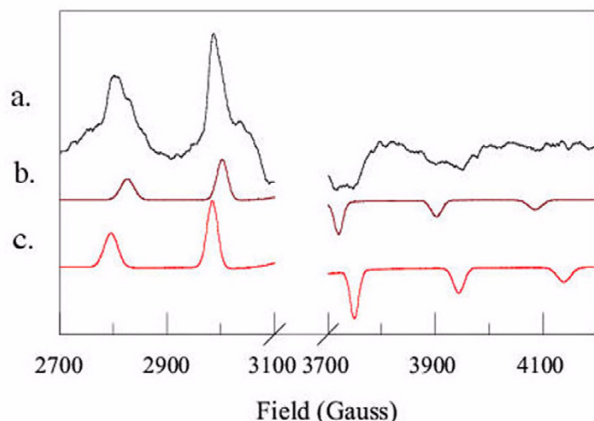
There are two sets of equatorial ligands that can fit well the EPR species D1 according to Eq 1 (Table 2). One set includes two equatorial oxygen from two water molecules, one equatorial oxygen from a carboxyl group or phosphate, and one equatorial nitrogen from an amino group. The other set contains one equatorial oxygen from water and three equatorial oxygens from carboxyl groups or phosphate. The best fit for the EPR species D2 to eq 1 is one equatorial oxygen from a hydroxyl group and three equatorial oxygens from carboxyl groups or phosphate.

#### EPR characteristics of sCD39 bound $\text{VO}^{2+}$ -ATP

In order to capture the bound  $\text{VO}^{2+}$ -EPR signal before the enzyme completely turned over, sCD39 and  $\text{VO}^{2+}$ -ATP were mixed on ice, immediately transferred into the EPR tube and frozen. The entire process took about 15 seconds. The parallel portion of the collected  $\text{VO}^{2+}$ -EPR spectrum is shown in Figure 5a.  $\text{VO}^{2+}$ -ATP complex

bound to sCD39 produced an EPR spectrum with  $A_{||}$  of 489.5 MHz and  $g_{||}$  of 1.9455, which corresponded to species D2 (Fig. 5b). The signal intensity from the bound  $\text{VO}^{2+}$ -nucleotide complex accounted only for 7.5% of that of species T from bound non-hydrolysable  $\text{VO}^{2+}$ -ADPNP complex.

The same sample made from mixing  $\text{VO}^{2+}$ -ATP and sCD39 was incubated at room temperature for 30 minutes, then the  $\text{VO}^{2+}$ -EPR spectrum was generated as shown in Figures 4c and 4d. The EPR parameters derived from this  $\text{VO}^{2+}$ -EPR spectrum were 489.5 MHz for  $A_{||}$  and 1.9455 for  $g_{||}$  respectively, which is consistent with species D2. However, the signal intensity decreased about 37.5 fold compared to that obtained before room temperature incubation.



**Figure 3**

Parallel regions of  $\text{VO}^{2+}$ -EPR spectrum of  $\text{VO}^{2+}$ -AMPCP bound to sCD39. a. One mole equivalent of  $\text{VO}^{2+}$ -AMPCP was added to 6.5 mg of sCD39 prepared as in Experimental Procedures. EPR conditions were as follows: field modulation frequency, 100 kHz; modulation amplitude, 0.97 G; receive gain,  $2 \times 10^4$ ; sweep time 5.243 s; time constant 1.28 ms; micropower, 1.0 mW; microwave frequency 9.40677 GHz, 1600 scans; temperature; 125 K. b. Simulated spectrum was generated by the program QPOWA with the experimental conditions above and  $A_{||} = 521.78$  MHz,  $g_{||} = 1.9370$  for Species D1. c. Simulated spectrum was generated by the program QPOWA with the experimental conditions above and  $A_{||} = 490.01$  MHz,  $g_{||} = 1.9435$  for Species D2.

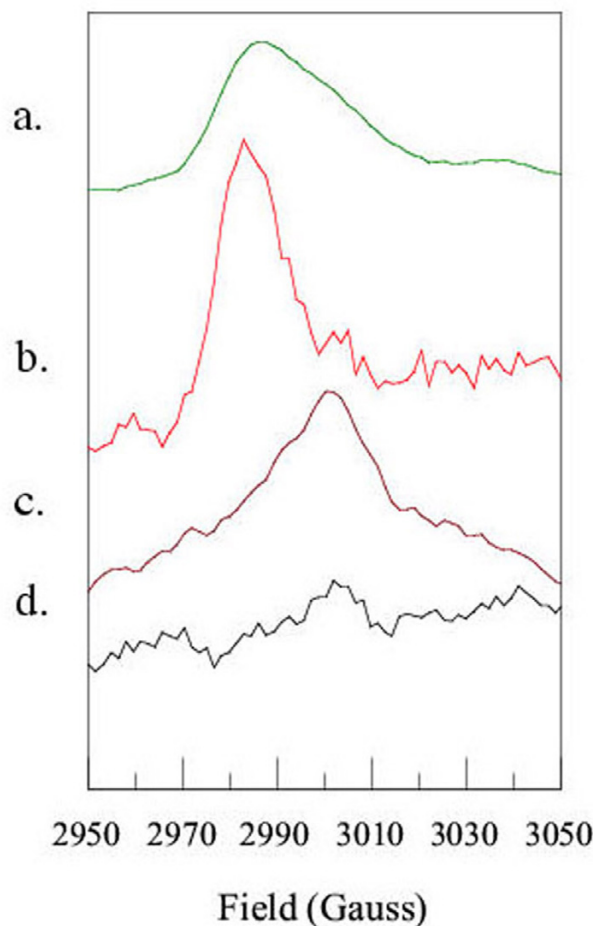
#### Free $\text{VO}^{2+}$ binding to sCD39 characterized by CD-EPR

Like other metals ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ), free  $\text{VO}^{2+}$  inhibited the nucleotidase activities of sCD39 at high concentration (data not shown).  $\text{VO}^{2+}$  in the absence of any nucleotides was added to sCD39 at 1:1 molar ratio. The parallel transitions of bound  $\text{VO}^{2+}$ -EPR spectrum are shown in Figure 6. The features derived from the  $\text{VO}^{2+}$ -EPR spectrum were 486 MHz for  $A_{||}$  and 1.946 for  $g_{||}$ , which was designed as species V (Fig. 6b). The signal intensity of bound  $\text{VO}^{2+}$  accounted for 20.3% of that from the bound  $\text{VO}^{2+}$ -ADPNP complex.

The best fit of equatorial ligands for species V according to eq 1 is two equatorial oxygen from hydroxyl groups and another two equatorial oxygen from two water molecules.

#### Discussion

Vanadyl has been used to estimate the types of groups that serve as metal-ligands in  $F_1$ -ATPase and other enzymes [16,18,19,21] because the  $g$  and  $A$  tensors of the  $^{51}\text{V}$  hyperfine couplings are approximately a linear combination of tensors from each type of group that contributes an equatorial ligand [24,27]. By studying the EPR spectra of bound  $\text{VO}^{2+}$  in the presence of different nucle-

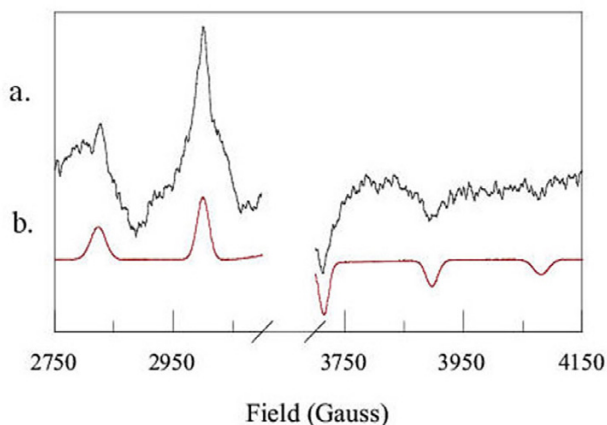


**Figure 4**

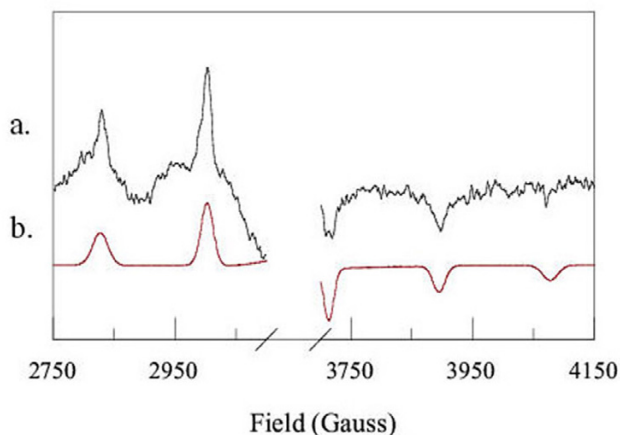
Parallel  $-5/2_{||}$  region of  $\text{VO}^{2+}$ -EPR spectra in the presence of different nucleotides and conditions. a. One equivalent of a 1 : 1 mole ratio of  $\text{VO}^{2+}$ -AMPPCP was added to sCD39, and incubated on ice for 5 minutes. b. The sample from a was thawed and incubated at room temperature for 30 minutes before collecting data again. c. One equivalent of a 1 : 1 mole ratio of  $\text{VO}^{2+}$ -ATP was added to sCD39, and frozen immediately in liquid nitrogen. d. The sample from c was thawed and incubated at room temperature for 30 minutes before collecting data again.

otides, we show that the interaction of soluble CD39 with ATP is different from that with ADP.

It is not surprising that  $\text{VO}^{2+}$  can functionally replace  $\text{Ca}^{2+}$  in the hydrolysis of both ATP and ADP by soluble CD39, although the enzymatic activity is about 25% of that with  $\text{Ca}^{2+}$  as the cofactor, since  $F_1$ -ATPase also hydrolyzes ATP at a decreased rate when  $\text{VO}^{2+}$  replaces  $\text{Mg}^{2+}$ [21].

**Figure 5**

Parallel regions of  $\text{VO}^{2+}$ -EPR spectrum of  $\text{VO}^{2+}$ -ATP bound to sCD39. a. One mole equivalent of  $\text{VO}^{2+}$ -ATP was added to 5.7 mg of sCD39 prepared as in Experimental Procedures. EPR conditions were as follows: field modulation frequency, 100 kHz; modulation amplitude, 0.97 G; receive gain,  $2 \times 10^4$ ; sweep time 5.243 s; time constant 1.28 ms; micropower, 1.0 mW; microwave frequency 9.40658 GHz, 3000 scans; temperature; 125 K. b. Simulated spectrum was generated by the program QPOWA with the experimental conditions above and  $A_{\parallel} = 489.5$  MHz,  $g_{\parallel} = 1.9455$  for Species D2.

**Figure 6**

Parallel regions of  $\text{VO}^{2+}$ -EPR spectrum of free  $\text{VO}^{2+}$  bound to sCD39. a. One mole equivalent of free  $\text{VO}^{2+}$  was added to 2.6 mg of sCD39 prepared as in Experimental Procedures. EPR conditions were as follows: field modulation frequency, 100 kHz; modulation amplitude, 0.97 G; receive gain,  $2 \times 10^4$ ; sweep time 5.243 s; time constant 1.28 ms; micropower, 1.0 mW; microwave frequency 9.40798 GHz, 3000 scans; temperature; 125 K. b. Simulated spectrum was generated by the program QPOWA with the experimental conditions above and  $A_{\parallel} = 486.00$  MHz,  $g_{\parallel} = 1.9460$  for Species V.

The EPR features of  $\text{VO}^{2+}$  are able to reveal some details about how CD39 hydrolyzes ATP and ADP. A single EPR feature, species T, was observed when ADPNP (a non-hydrolyzable analog of ATP) complexed with  $\text{VO}^{2+}$  was bound to sCD39, which is consistent with the presence of only one nucleotide binding site [25]. The  $g$  and  $A$  tensors derived from species T are 1.9410 and 504.25 MHz respectively, which can be fitted best with one amino group and three groups combined from carboxyl and phosphate groups as the equatorial ligands of the bound  $\text{VO}^{2+}$  on sCD39. In accordance with metal-ATP complex coordination on other enzymes that hydrolyze ATP, like  $F_1$ -ATPase [22,28], the  $\gamma$ - and  $\beta$ -phosphate of ATP most likely bind to  $\text{VO}^{2+}$  while the third carboxyl group is contributed by a side-chain of aspartate or glutamate of sCD39. It is not unusual for the  $\epsilon$ -amino group of lysine to coordinate with metals in enzymes. It has been reported that the amino group serves as one of  $\text{VO}^{2+}$  equatorial ligands in  $CF_1$ -ATPase [21], pyruvate kinase [19,20], AdoMet synthetase [17,18], and carboxypeptidase [16]. Thus one amino group from lysine, one carboxyl group from aspartate or glutamate, and two oxygens from the phosphates of ADPNP serve as the equatorial ligands of sCD39 bound  $\text{VO}^{2+}$  in the presence of ADPNP.

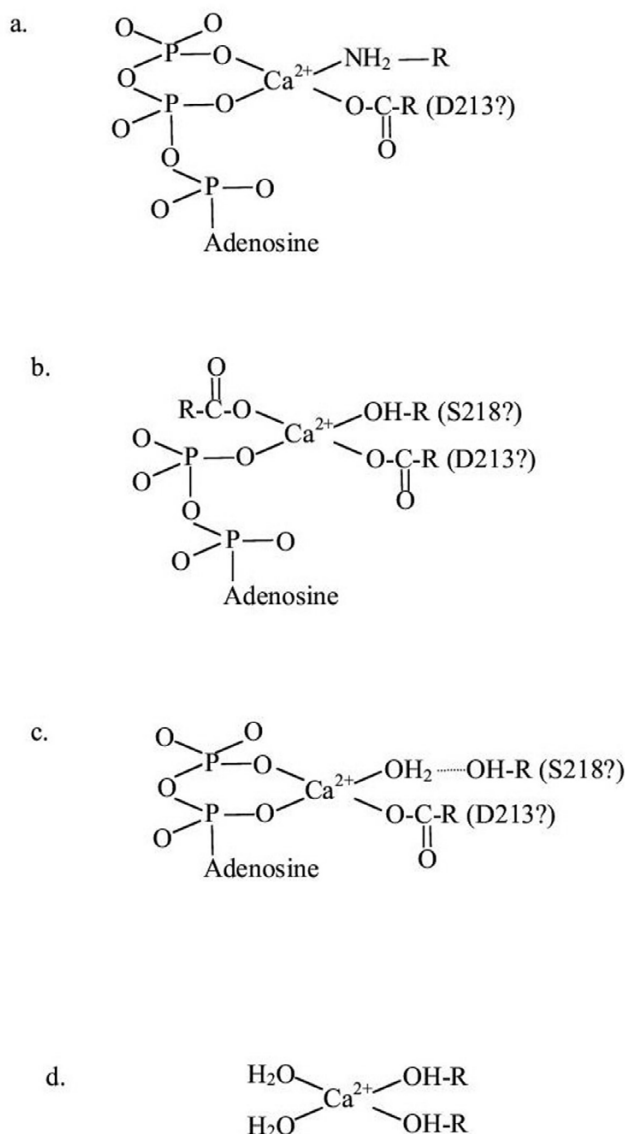
In the presence of AMPCP, bound  $\text{VO}^{2+}$  produced two EPR features, species D1 and species D2 that are separated by about 30 MHz. As we have reported that sCD39 releases intermediate ADP before ADP is further cleaved during ATP hydrolysis [25], sCD39 probably has two conformations that bind metal-ADP complexes, one is the conformation that releases the ADP intermediate, and another that recruits intermediate ADP back to the enzyme for further hydrolysis to AMP. However, intact CD39 does not release intermediate ADP during ATP hydrolysis, suggesting that there is only one ADP binding site on each CD39 monomer in the intact protein [2,25]. The two EPR species observed with  $\text{VO}^{2+}$ -AMPCP probably correspond to the two different conformations of bound ADP at the same catalytic site on sCD39. The signal intensity of the bound  $\text{VO}^{2+}$ -AMPCP EPR spectrum indicates that species D1 is dominant over species D2. In order to further assign species D1 and D2 to the two different conformations, two experiments were done (Fig. 5). Incubation of sCD39 with  $\text{VO}^{2+}$ -AMPCP at room temperature resulted in a dramatic decrease of the intensity of species D2, while the signal intensity of species D1 remained unchanged. These data indicate that  $\text{VO}^{2+}$ -AMPCP was released from the conformation corresponding to species D2; however, the conformation corresponding to species D1 still had bound  $\text{VO}^{2+}$ -AMPCP. More evidence for two conformations of the enzyme was obtained from the EPR spectra of bound  $\text{VO}^{2+}$ -ATP. No species T was found presumably because ATP was converted to ADP before the sample was frozen. Only species D2 was

observed and its intensity decreased as the incubation time was prolonged. We suggest that species D2 corresponds to the conformation that releases ADP as an intermediate product and species D1 corresponds to the conformation that binds ADP as a substrate. The lower signal intensities of species D1 and D2 compared to that of species T suggest that the affinity of sCD39 for ADP or its analog AMPCP is lower than that for the ATP analog ADPNP, which is consistent with the result that only ATP analogs were detected on sCD39 [25].

The calculated  $g_{||}$  and  $A_{||}$  values that best matched the experimental values for species D2 suggest that one hydroxyl group and three oxygens derived from carboxyl groups and phosphates are the equatorial ligands of bound  $VO^{2+}$ -ADP. Since the conformation corresponding to species D2 is found in the presence of ATP and is likely to be the conformation that releases bound  $VO^{2+}$ -ADP, it is likely that the  $VO^{2+}$  ligands are one phosphate and two carboxyl groups [25]. When ADP is the substrate and generates species D1, one water molecule and a combination of three groups between carboxyl groups and phosphates serve as the equatorial ligands of bound  $VO^{2+}$  on sCD39. The probable combination of carboxyl groups and phosphates for species D1 is one carboxyl group and two phosphates since  $VO^{2+}$  complexes ADP through two phosphates before  $VO^{2+}$ -ADP is bound to the enzyme.

The site directed mutagenesis studies on CD39 and other members of the CD39 family give some hints about the possible residues that serve as metal ligands at the catalytic site of sCD39. The changes of D62 on ACR1, E174 on ACR3, D213 (D219 in HB6) and S218 on ACR4 dramatically decrease both ATPase and ADPase activities of CD39 [12,15]. Figure 7 summarizes the possible coordination of  $Ca^{2+}$  from the data of species T, species D1, and species D2 in the different situations of sCD39 catalysis. The catalytic base attack results in cleavage of the  $\gamma$ -phosphate of ATP, and one carboxyl group replaces the  $\gamma$ -phosphate as a metal ligand (from species T to species D2), which is accompanied by a swap of an amino group with a hydroxyl group (S218?). This hydroxyl group (S218?) probably interacts with the water molecule through hydrogen bond in the conformation corresponding to species D1 to hydrolyze ADP. The constant carboxyl group that appears in all conformations of sCD39 hydrolysis is likely contributed by D213 since it is close to S218.

The results presented here also provide an explanation to the free metal inhibition of CD39 catalytic activity. Free  $VO^{2+}$  binds to sCD39 through two hydroxyl groups and two water molecules that are hydrogen bonded to other residues of sCD39. Once free  $VO^{2+}$  occupies the catalytic



**Figure 7**

Summary of the ligands at the metal coordination sites of sCD39 during the catalytic reaction. The types of groups shown as equatorial ligands were derived from the best fits of  $^{51}V$  hyperfine values from Table 2. a. The metal ligands in the presence of ADPNP (Species T) represent the conformation of ATP at the beginning of hydrolysis by sCD39. b. The metal coordination sites in the presence of AMPCP (Species D2) show the arrangement of the catalytic site prior to release of ADP as an intermediate product. c. The metal coordination sites in the presence of AMPCP (Species D1) show the conformation of the catalytic site ready to hydrolyze ADP. d. Metal coordination in the absence of nucleotide (Species V) showing the occupancy of the active site by free metal with exclusion of nucleotide and consequent inhibition of sCD39.

site, the enzyme has to either release the metal or correct the conformation before the substrates are recruited properly.

### Conclusions

VO<sup>2+</sup> can functionally substitute for Ca<sup>2+</sup> as a cofactor for sCD39. Four different EPR spectra are obtained for VO<sup>2+</sup> bound in the presence of different nucleotides and in the absence of nucleotide. The protein ligands for VO<sup>2+</sup> in the presence of ATP are suggested to be carboxyl and amino groups, while those in the presence of ADP are probably carboxyl and hydroxyl groups. The mechanism of sCD catalysis is discussed. These results will provide guides for further studies of the catalytic mechanism of NTPDases.

### Materials and Methods

#### Reagents

ATP, ADP, ADPNP, AMPCP were purchased from Sigma (St. Louis, MO). Zeocin, High-Five medium were purchased from Invitrogen (Carlsbad, CA).

#### Cell culture and preparation of soluble CD39

sCD39 transfected stable HighFive™ insect cells were cultured as described by Chen and Guidotti [25]. Soluble CD39 were purified as described [25] with some modifications. After concanavalin A-Sepharose 4B and nickel affinity column chromatography, the ammonium sulfate precipitated sCD39 was collected and resuspended in about 50 µl of 40 mM Tris-HCl (pH7.5). This sample was loaded on a Superose-12HR gel filtration column from Pharmacia Biotech equilibrated with 40 mM Tris-HCl (pH7.5). The fractions containing the major peak were collected, and the solvent was changed to 20 mM Hepes (pH8.0), 120 mM NaCl, 5 mM KCl with an YM30 centrifuge from Millipore. The final volume of the sample was around 200 µl, and the concentration of sCD39 was around 0.1 mM.

Concentrations of proteins were determined using D<sub>C</sub> Protein Assay from BIO-RAD using the provided protocol.

#### Nucleotidase activity assay and nucleotide separation by HPLC

The reactions were carried out in 20 mM HEPES-Tris (pH 7.0), 120 mM NaCl, and 5 mM KCl; they were started by adding nucleotides at 37°C. After incubation for 15 minutes, the reactions were stopped with 2% perchloroacetic acid

Nucleotides were separated by HPLC on an anion exchange column (a 10 × 0.46 mm SAX column from Rainin Instruments) based on the method of Hartwick and Brown [29]. The low concentration buffer (A) was 0.08

M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> (pH3.8), and the high concentration buffer (B) was 0.25 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> (pH4.95) with 8 mM KCl. The gradient used was 4 min, 0–2.5% (B); 26 min, 2.5–25% (B). Equilibration was done with buffer (A) for 10 minutes, and the flow rate was 1 ml/min.

#### Preparation of VO<sup>2+</sup> solution

Vanadyl and nucleotide solution were prepared according to Houseman et al. [21]. Dissolved molecular oxygen was removed from solutions by purging with dry nitrogen gas. Stock vanadyl and nucleotide solution were thawed on ice, and mixed at 1:1 molar ratio by vigorous stirring. Then VO<sup>2+</sup>-nucleotide complexes were added to purified sCD39 at 1:1 molar ratio, mixed, and incubated for 5 minutes on ice before they were transferred into EPR tubes. Once the samples were in EPR tubes, they were immediately frozen in liquid nitrogen, and stored in liquid nitrogen before using.

#### EPR Measurement

CW-EPR experiments were carried out at X-band (9 GHz) using a Bruker 300E spectrometer with a TE102 rectangular standard cavity and a liquid nitrogen flow cryostat operating at 150 K. Simulations of these EPR spectra were accomplished with the computer program QPOWA [30,31].

To estimate the types of groups that serve as equatorial ligands to VO<sup>2+</sup> in each condition, the observed values of A<sub>||</sub> derived from simulation of the EPR spectrum by QPOWA were compared with the coupling constants obtained from model studies [24,32] using:

$$A_{||\text{calc}} = \sum n_i A_{||i} / 4$$

where i represents the different types of equatorial ligand and donor groups, n<sub>i</sub> (=1–4) is the number of ligands of type i, and A<sub>||i</sub> is the measured coupling constant for equatorial donor group i [24]. Similar equations were used to calculate g<sub>||</sub> from a given set of equatorial ligands for comparison with those derived experimentally.

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