BioMetals 1997, **10**, 291–298

Binding of vanadate to human erythrocyte ghosts and subsequent events

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Received 5 November 1996; accepted for publication 5 March 1997

Spectroscopic techniques were used to investigate the interaction between vanadate and human erythrocyte ghosts. Direct evidence from ^{51}V nuclear magnetic resonance (NMR) studies suggested that the monomeric and polymeric vanadate species may bind to the anion binding sites of band 3 protein of the erythrocyte membrane. The results of ^{51}V NMR studies and the quenching effect of vanadate on the intrinsic fluorescence of the membrane proteins indicated that in the low concentration range of vanadate (<0.6 mM), monomeric vanadate binds mostly to the anion sites of band 3 protein with the dissociation constant close to 0.23 mM. The experiments of sulfhydryl content titration by the method of Ellman and residue sulfhydryl-labeled fluorescence spectroscopies clearly displayed that vanadate reacts directly with sulfhydryl groups. The appearance of the anisotropic election spin resonance (ESR) signal of vanadyl suggests that a small (*c.* 3%) amount of vanadate was reduced by sulfhydryl groups of membrane proteins. The fluidity and order of intact ghost membrane were reduced by the reaction with vanadate, as shown by the ESR studies employing the protein- and lipid-specific spin labels. It was concluded that although vanadates mainly bind to band 3 protein, a minor part of vanadate may oxidize the residue sulfhydryl groups of membrane proteins, and thus decrease the fluidity of erythrocyte membrane.

Keywords: band 3 protein, erythrocyte membrane, membrane fluidity, sulfhydryl groups, vanadate

Introduction

Vanadium is outstanding among all the metals in the diversity of its biological effects and biological functions (Rehder 1991). Vanadate and several other vanadium compounds were shown to behave as insulin-like agents (Crans *et al.* 1995, Orvig *et al.* 1995), cardiovascular function effectors (Chasteen 1983), microtubule and microfilament stabilizers (Combeau & Carlier 1988), the activator or inhibitors of a variety of enzymes (Stankiewicz & Tracy 1995, Stankiewicz *et al.* 1995), the promoters of lipid peroxidation (Keller *et al.* 1988) and also as the essential metal for tunicates (Oltz *et al.* 1988), etc. Several biological effects have been discussed on the basis of the analogy of vanadate with phosphate (Chasteen 1990), but some others are not interpretable as such. Evidently, since vanadate and vanadyl ions are interconvertible in physiological conditions, vanadium's biological behaviour is likely the integration of those caused by both vanadate and vanadyl ions. We considered that the biological effects of various inorganic ions might be traced to their analogy with the essential ions Ca^{2+}, phosphate and also Fe^{2+}/Fe^{3+}. The analogy of vanadate with phosphate and that of the vanadyl ion with both Ca^{2+} and Fe^{2+} are likely the foundation of all the biological effects of vanadium (Zhang 1996).

It was shown that the vanadate ions are transported into the erythrocyte rapidly through anion channels (Heinz *et al.* 1982) and reduced to the

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vanadyl ion within the cells mainly by the intracellular reductant glutathione (Macara *et al.* 1980). Although the effects of vanadate on erythrocytes might involve those of vanadyl, several points are not so clear. The anion transportation by band 3 is affected by a binding-releasing process. We are not clear on the way by which the vanadate ions enter the cells and are reduced. We should ask where the vanadate ions are reduced: in the membrane or in the cytosol? If the vanadyl ions are produced in the cytosol and bind to cystolic proteins, such as hemoglobin, rapidly, how does the vanadyl ion induce Fenton-like reactions? The present work has been focused on the fate of vanadate ions bound to human erythrocyte membrane. ^{51}V NMR was used to study the binding of vanadate to band 3 protein, and vanadyl–EPR spectroscopy, sulfhydryl content titration and the spin-labeling method of erythrocyte ghosts were used to probe the subsequent changes of the vanadate and the membrane.

Materials and methods

Materials

Human red blood cells (Red Cross Blood Center, Beijing, China); vanadium pentoxide (99.9%, Shanghai Chemical Co., China); *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) were purchased from Aldrich (Milwaukee, WI, USA); 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), *N*-(1-pyrenyl)maleimide (NPM), 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) and 5-doxylstearic acid (5-NS) were all from Sigma (St. Louis, MO, USA) and used without further purification. The stock solution of sodium vanadate was prepared by dissolving vanadium pentoxide in 2M NaOH, after which the pH was adjusted to 8.0 with 2M HCl. The vanadium concentration was determined by means of EDTA titration.

Erythrocyte ghosts

The ghosts were prepared from fresh blood. The red blood cells were washed three times with the isotonic solution containing 135 mM NaCl and 15 mM HEPES (pH 7.4), and packed by centrifugation to a packed cell volume of 95%. The cells were lysed in 20 volumes of 5 mM HEPES (pH 8.0) at 0°C. The leaky ghosts were subsequently concentrated by centrifugation at 17 000 rpm for 20 min, washed twice in the same buffer, and then frozen. The intact ghosts were prepared by incubating the leaky ghosts in three volumes of the isotonic HEPES solution at 4°C overnight, and packed by centrifugation at 17 000 rpm for 12 min. The protein content in the final ghost pellets was assayed by the modified Lowry method (Lowry *et al.* 1951).

^{51}V NMR spectroscopy

The spectra were scanned on a Varian XL-400 NMR spectrometer with the broadband facility operating at 20 ± 0.5°C. The spectra were obtained at 105 MHz, 90° pulse widths and 0.10 s acquisition time. The average number of scans was 10 000. A line-broadening factor of 20 Hz was applied to all spectra before Fourier transformation to the frequency domain. Signal/noise ratio was controlled at a constant value; the line widths at half maximum ($LW_{1/2}$) for the ^{51}V resonances were determined by fitting the signals to Lorentzian line shapes. The peaks of ^{51}V spectra were assigned according to reference (Crans 1994). The chemical shifts were determined with $VOCl_3$ as an external standard.

The samples for ^{51}V NMR measurements were prepared by mixing the leaky ghost pellets and vanadate stock solution in 5 mM HEPES buffer (pH 8.0) to keep the final concentration of vanadate as 1.0 mM or 4.0 mM but varying the membrane protein concentrations in the approximate range of 0–3.0 mg ml^{-1}. After incubating the samples for 4 h at 4°C, the NMR spectrum was scanned; 100 μl 30 mM DNDS were then added and a second spectrum was obtained.

Fluorescence spectroscopic studies

All the fluorescence measurements were conducted on a Shimadzu RF-540 fluorescent spectrometer.

Intrinsic fluorescence of membrane proteins 100 μl vanadate solutions with varying vanadate concentration were mixed with each 3.0 ml suspension of leaky ghost (3.2 mg ml^{-1} protein) in 5 mM HEPES buffer (pH 8.0). After incubation at either 20 ± 0.5°C or 37 ± 0.5°C for 3 h, the fluorescence intensity at 335 nm was recorded with an excitation wavelength of 285 nm.

NPM-labeled fluorescence. Into the 3.0 ml of the leaky ghost suspensions (3.2 mg ml^{-1} protein), 100 μl vanadate solution of various concentrations in 5 mM HEPES buffer (pH 8.0) were added, respectively. The mixtures were incubated at 20 ± 0.5°C for 3 h, then 25 μl of 1.0 M NPM in dioxane were added and the mixtures incubated at 4°C for 24 h to accomplish the labeling of the proteins. The emission intensity was recorded at 375 nm with an excitation wavelength of 342 nm.

ESR studies

All ESR spectra were recorded at room temperature (23 ± 0.5°C) in 1 mm outer diameter (o.d.) quartz tubes on a Bruker ESR 300 spectrometer with computerized data acquisition. The spectrometer was operating at X-band and was calibrated using a powder sample of 2,2-bis(4-*tert*-octylphenyl)-1-picrylhydrazyl (2.0037g).

Characterization of the formation of the vanadyl–membrane complex. The concentration of the vanadyl–ghost complex was estimated from the area under the specific ESR absorption peak as described previously (Chasteen 1981). The samples were prepared by mixing vigorously the vanadate stock solution with the leaky ghost suspension in 5 mM HEPES (pH 8.0). The total concentration of vanadium was 1.0 mM, and that of membrane protein was 3.4 mg ml^{-1}. The mixed suspensions were incubated at room temperature under sterilized but aerobic conditions.

MAL-6 and 5-NS spin-labeled spectra. The intact ghosts were spin-labeled with either a protein-specific spin label, MAL-6 (Jay *et al.* 1987), or a lipid-specific spin label, 5-NS (Jay *et al.* 1987), as previously described (Wyse & Butterfield 1989, Chen *et al.* 1992). The solutions of vanadate in various concentrations (Table 2) in isotonic HEPES (pH 7.4) at a total volume of 750 μl were mixed with 250 μl spin-labeled intact ghost suspension (4.3 mg ml^{-1} protein). The mixed suspensions were incubated at 37°C for 0.5 h, then concentrated by centrifugation at 4000 rpm for 20 min. They were then washed with vanadate solution of the same concentration in isotonic HEPES buffer until no ESR signal of the free MAL-6 or 5-NS was detected in the supernatant.

From the MAL-6 spin-labeled ESR spectra, the ratio of the ESR spectral amplitude of the $M_I = +1$ low-field weakly immobilized line (W) and that of the $M_I = +1$ low-field strongly immobilized line (S) (the W/S ratio) was obtained, as shown in reference (Wyse & Butterfield 1989). Based on 5-NS spin-labeled ESR spectra, the order parameter S was calculated by the following equation (Chen *et al.* 1992):

$$S = 0.568\ (A_{\parallel} - A_{\perp})/(A_{\parallel} + 2A_{\perp})/3$$

where $A_{\parallel}$ and $A_{\perp}$ are parallel and perpendicular parts of the hyperfine splitting corresponding to one-half the separation of the outer peaks and the inner peaks in a spectrum, respectively.

The sulfhydryl content measurements

The solutions of vanadate of various concentrations (each 50 μl) were mixed with 1.0 ml leaky ghost suspension (50 μg ml^{-1} protein) in 5 mM HEPES buffer (pH 8.0). The mixtures were incubated at 37 ± 0.5°C for 12 h, after which the thiol group content was determined according to the method of Ellman (1959).

Results and discussion

The binding of vanadate to membrane proteins

The aqueous chemistry of vanadate is complicated by its oligomerization and reduction. Vanadate usually exists as the mixture of its monomer (V_1), dimer (V_2), tetramer (V_4) and pentamer (V_5) in the range of micromolar to millimolar concentration in neutral aqueous solutions. The distribution is dependent on the total vanadium concentration, pH and ionic strength of the solution (Crans 1994).

In the present work, the binding of vanadate to erythrocyte membrane was studied by means of the ^{51}V NMR method. The ^{51}V NMR spectrum of 4.0 mM vanadate in 5 mM HEPES (pH 8.0) solution is featured by four resonances (Figure 1) corresponding to monomeric (V_1), dimeric (V_2), tetrameric (V_4) and a very small amount of pentameric (V_5) vanadates. After incubation with the leaky ghost suspension for 4 h, all the resonances were broadened and shifted toward high field. Among them, the line-broadening (49 Hz) and chemical shifting (5 ppm) of V_1 were the most significant. The subsequent addition of DNDS enables the $LW_{1/2}$ to recover toward its original value. DNDS binds specifically to the anion binding site of band 3 with extremely high affinity (K_d = 6.4 μM) (Falke & Chan 1986), therefore the difference between the $LW_{1/2}$ values with and without DNDS reflects the line-broadening effect due to vanadate binding to the anion sites of band 3 protein. The spectra given in Figure 1 indicate that all four vanadate species are able to bind to these sites. However, since the line-broadening and shifting are not inhibited completely

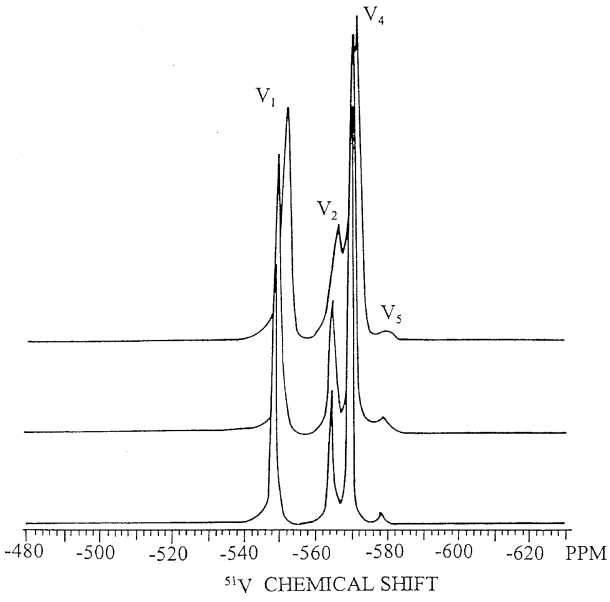

Figure 1. ^{51}V NMR spectra of 4.0 mM vanadate in 5 mM HEPES solution at pH 8.0 and 20°C, without leaky ghosts (bottom), with leaky ghosts at 3.0 mg ml^{-1} protein concentration (top), and with ghosts and imM DNDS (center). V_1, V_2, V_4 and V_5 stand for vanadate monomer, dimer, tetramer and pentamer, respectively.

by DNDS, the reactions of vanadate with other membrane-bound molecules cannot be excluded.

When the vanadate concentration was held constant at 1.0 mM (in order to keep the vanadate mainly as monomer in 5 mM HEPES at pH 8.0) and the membrane protein concentration of the leaky ghosts was varied, the line-broadening induced from the binding of monomeric vanadate to the anion sites of band 3 and to other sites were both found to increase linearly with the ghost protein concentration (Figure 2); the slopes were estimated to be 31.8 ± 2.6 Hz $mg^{-1}ml^{-1}$ and 4.5 ± 0.3 Hz mg^{-1} ml^{-1}, respectively. The results indicate that the line-broadening of V_1 resonance is attributed mainly (86%) to its binding to the specific anion sites of band 3.

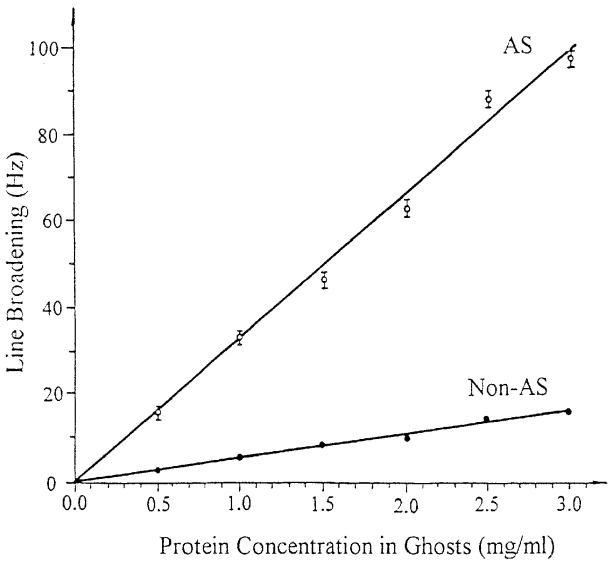

Figure 2. The anion sites (AS) of band 3 specific and non-AS line-broadening of the ^{51}V resonance as a function of human erythrocyte ghost membrane protein concentration. The solutions contain: 1.0 mM vanadate and leaky ghosts with various protein concentrations in 5 mM HEPES buffer (pH 8.0), temperature 20°C.

The quenching effect of vanadate on the intrinsic fluorescence of the membrane proteins

As shown in Figure 3, the addition of vanadate into leaky ghost suspension at 20°C induced quenching of the intrinsic fluorescence of membrane proteins. The concentration-dependent quenching effect indicates the interactions between vanadate and membrane proteins. No quenching effect was observed in the titrations with either phosphate or chloride anions under the same conditions. It can be deduced that by contrast with phosphate and chloride the binding of vanadate with membrane proteins results in conformational changes of the ghost membrane proteins; a similar case was reported by Highsmith *et al.* (1985), in which the high-affinity and low-affinity vanadate binding to membrane bound Ca^{2+}-ATPase of sarcoplasmic reticulum caused a conformational change of the protein.

In general, fluorescence quenching falls into two categories (Lakowicz 1983): dynamic quenching and static quenching. These can be distinguished by their different temperature dependence, or by lifetime measurements. Both static and dynamic quenching of fluorescence can be described on the basis of the Stern–Volmer equation (Lakowicz 1983):

$$F_0/F = 1 + K[Q]$$

In this equation F_0 and F are the fluorescence intensities in the absence and presence of quencher respectively and $[Q]$ stands for the concentration of quencher. In the case of dynamic quenching, K is the Stern–Volmer quenching constant K_D, while in the case of static quenching, it is the association constant K_S.

We obtained the fluorescence quenching data by means of vanadate titration at 20°C and 37°C, respectively. As shown in Figure 4, the quenching

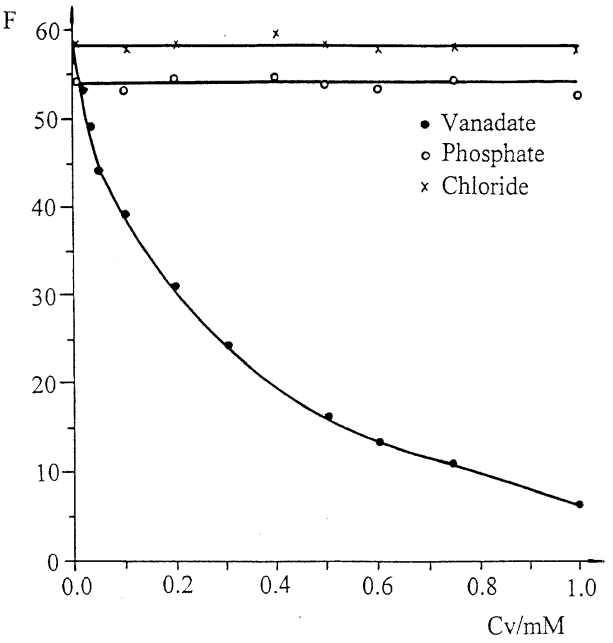

Figure 3. The quenching curves of vanadate, phosphate and chloride to the intrinsic fluorescence of erythrocyte membrane at 20°C. Solutions: the leaky ghosts (3.2 mg ml^{-1} protein) and various concentrations of the three anions in 5 mM HEPES (pH 8.0).

ability of vanadate at 37°C was lower than that at 20°C. This indicates that it is mainly a static quenching and implies the formation of static complexes of vanadate–membrane because increased temperature is likely to destablilize the complexes and thus lead to lower values of the static quenching constants. When the concentration of vanadate was changed in the approximate range of 0–0.6 mM, the Stern–Volmer plots at both temperatures were linear, and the apparent formation constants of vanadate–membrane protein complexes at 20°C and 37°C were obtained as $K_{S,20} = 4.3$ mM^{-1} and $K_{S,37} = 2.2$ mM^{-1} from the linearities, respectively. Since vanadate exists mainly as monomer in 5 mM HEPES solution (pH 8.0) in the low concentrations, and binds mostly to the anion binding sites of band 3, it is reasonable to suggest that this value approximates the actual value of the binding constant of V_1 to anion binding sites. Thus, the dissociation constant of vanadate binding to the anion binding sites of band 3 at 20°C was estimated to be $K_d = 1/K_s = 0.23$ mM. This estimated value is close to the dissociation constant (0.5 ± 0.1 mM) of vanadate binding to the anion exchanger of sarcoplasmic reticulum membrane determined by ^{35}Cl NMR studies (Vetter *et al.* 1991). To compare the K_d for vanadate with those for nitrate and chloride (6.9 mM and 74 mM) with human erythrocyte membrane at the same temperature (20°C) (Galanter & Labotka 1991), the binding strength decreases in the following sequence: DNDS >> vanadate > nitrate > chloride. When the concentration of vanadate was higher than 0.6 mM, the Stern–Volmer plots acquired an upward curvature. This feature indicates that the vanadate quenches the intrinsic fluorescence by collisional encounters in addition to complex formation at higher concentration (Lakowicz 1983).

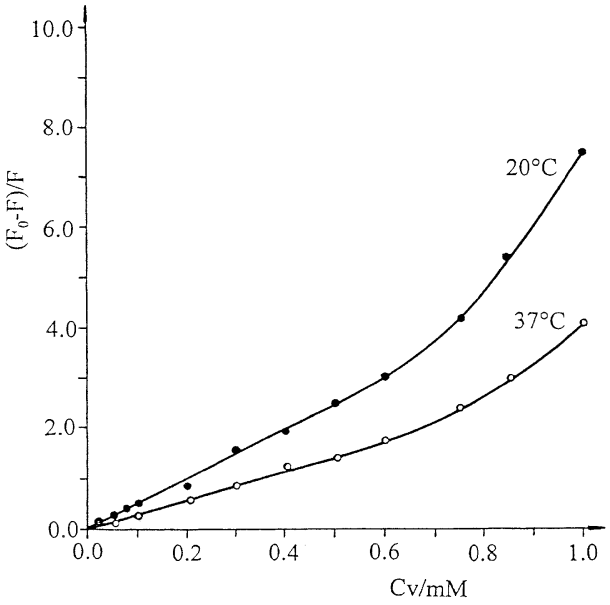

Figure 4. Stern–Volmer plots of vanadate quenching the intrinsic fluorescence of membrane proteins at 20 and 37°C, respectively.

The reduction of vanadate by membrane

The vanadate ion, as an oxyanion of a transition metal, differs from anions such as phosphate and chloride in several aspects. One outstanding feature of vanadate is its single-electron reduction. Many experiments have confirmed that vanadate can be reduced to vanadyl by sulfhydryl compounds such as glutathione and cysteine within the cells (Macara *et al.* 1980) or *in vitro* (Legrum 1986).

After incubation with the leaky ghost suspension for 40 h, the ESR spectrum is featured by a typical signal of a static distribution of randomly oriented vanadyl ions (Figure 5, bottom). The signal decreases upon further incubation (Figure 5, center), and disappears completely after one week (Figure 5, top). Meanwhile, the color of the solution incubated in the aerobic condition had changed from colorless to faint yellow. It should be noted that even at the time when the vanadyl signal was strongest (40 h), the vanadyl spin concentration was rather low, 31 ± 1 μM. These results indicate that only a small fraction of vanadate (3%) was reduced to vanadyl, and that almost all the vanadyl ions thus formed are complexed to certain macromolecules, which are

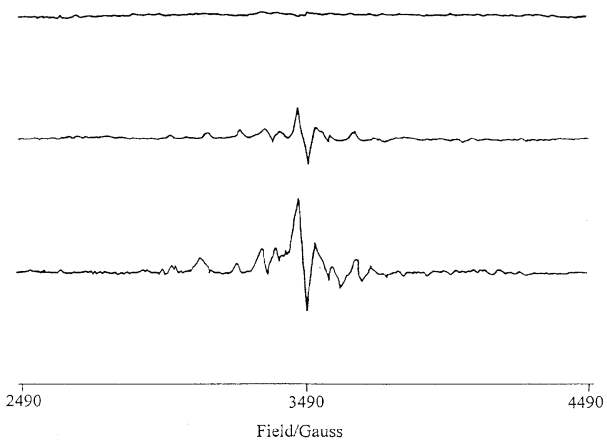

Figure 5. The ESR spectra of the mixed suspension of vanadate (1.0 mM) and leaky ghost (4.0 mg ml^{-1} membrane protein) in 5 mM HEPES solution (pH 8.0) after incubation for 40 h (bottom), 64 h (center) and 7 days (top) at room temperature (23 ± 0.5°C).

large enough to prevent the rotation (at 23°C) during a time comparable with the reciprocal of the spectral anisotropy (in frequency units). These results are also consistent with the possibility that the protein-bound vanadate was reduced *in situ* by the sulfhydryl groups of membrane protein to give a vanadyl–thioester complex and was eventually reoxidized by oxygen or the lipid peroxides to the vanadate complex, since the vanadyl signal tends to disappear upon standing.

The sulfhydryl groups of membrane proteins were oxidized partially by vanadate

By means of Ellman's method, the free sulfhydryl content of erythrocyte ghosts before and after the incubations with various concentrations of vanadate was determined. The results (Table 1) indicate that with increasing concentrations of vanadate, the sulfhydryl content of membrane protein decreases. When the concentration reached 1.02 mM, the sulfhydryl content decreased to 23% relative to the reference value.

NPM is used as a fluorescent label. It is nonfluorescent in aqueous solution until it binds to sulfhydryl compounds. If the sulfhydryl sites have already been occupied by vanadate and the complexes formed are stable enough, NPM would not label these sulfhydryl sites. Hence, the NPM-labeled fluorescence intensity of erythrocyte membrane after incubation with vanadate may reflect the extent of interaction of vanadate with sulfhydryl groups of membrane proteins. The curve in Figure 6 shows that the fluorescence intensity of NPM-labeled leaky ghosts decreases with the increasing vanadate concentration in the incubation media. This indicates that vanadate reacts directly with the sulfhydryl groups on the membrane protein, and is consistent with the result of DTNB determination.

The results of thiol groups titration based on DTNB and NPM fluorescence clearly show that vanadate reacts with sulfhydryl groups directly. Since no other reducing agents were present in the experimental leaky ghost systems, the appearance of the anisotropic ESR signal of vanadyl ions complexed to membrane protein suggests that a small amount of vanadate was reduced by sulfhydryl groups of membrane protein. For the reduction of vanadate by thiols, a mechanism has been suggested

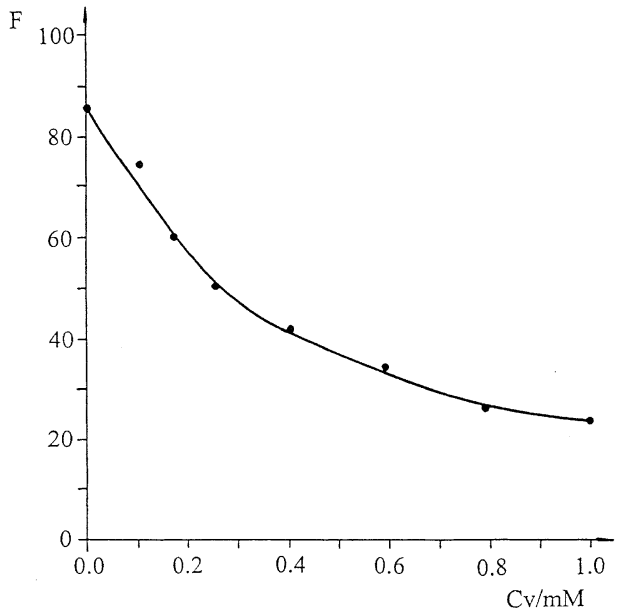

Figure 6. The relative fluorescent intensity of NPM-labeled leaky ghosts changes as a function of the vanadate concentration incubated previously. The solution conditions are given in the text, Materials and methods.

(Legrum 1986), including an intermediate vanadate thioester followed by its disproportionation to disulfide or reduction to vanadyl. The latter tends to be reoxidized to vanadate by peroxides and/or oxygen in neutral pH. The process from appearance to disappearance of the vanadyl ESR signal agrees well with these suggestions.

Membrane fluidity and order in membrane structure were reduced by the reaction between membrane and vanadate

The protein-specific spin label, MAL-6, binds covalently to membrane protein sulfhydryl groups. Previous ESR studies (Jay *et al.* 1987, Wyse & Butterfield 1989) confirmed that in an ESR spectrum of MAL-6 labeled erythrocyte membrane, the MAL-6 binding sulfhydryl sites fall into two categories, characterized by their different motilities: the weakly and strongly immobilized. The W/S ratio is extremely sensitive to the conformation change and the interactions among the membrane proteins, and also reflects the segmental motion of protein binding

Table 1. DTNB determination of the decrease in the sulfhydryl content of erythrocyte membrane by vanadate

Concentration of vanadate (mM)	0	0.138	0.275	0.683	1.02
Sulfhydryl content (nmol per mg protein ±SD)	73 ± 1	65 ± 1	63 ± 2	61 ± 2	56 ± 2

sites (for review see Butterfield 1982). After incubating the MAL-6 spin-labeled intact ghosts with various concentrations of vanadate, their ESR spectra were recorded. The calculated data of W/S ratio are listed in Table 2. The W/S ratio values decrease significantly with the increasing concentration of added vanadate (Table 2). This reflects that vanadate causes an increase of intra and/or intermolecular interactions of membrane proteins and dampens the segment motion and rotation of the proteins (Butterfield 1982, Wyse & Butterfield 1989). This may be due to the promoted oxidative cross-linking of sulfhydryl groups.

To gain an insight into whether such an interaction altered the order of membrane structure and motion of the lipid molecules, the lipids were labeled with 5-NS, the lipid-specific spin label, the order and motion of the membrane lipids were assessed by measuring $A_{\parallel}$ and $A_{\perp}$ and the order parameters were calculated (Chen *et al*. 1992). The order parameter reflects the motion state of the hydrocarbon chains of the fatty acids in the lipid bilayer. It is related to the anisotropism, amplitude and frequency of lipid molecular motion. As the lipid motion decreases, the order parameter increases toward unity. As shown in Table 2, the influences of vanadate on the order parameter values are concentration dependent. Under the experimental conditions, with increasing concentrations of vanadate, the order parameter increases, but not so significantly. This means that the interactions of vanadate with membrane result in decreased motility of lipid molecules.

Keller *et al*. (1988) reported that vanadyl is the active form of vanadium in initiating conjugated diene formation in micelles prepared from purified or partially peroxidized unsaturated fatty acids. Our previous experimental results (Chen *et al*. 1997) indicated that reactive-oxygen-species scavengers such as mannitol, ethanol and SOD do not inhibit the lipid peroxidation of erythrocyte membrane promoted by the vanadyl ions, but that EDTA does inhibit the action of vanadyl. It is speculated that vanadyl ions initiate lipid peroxidation mainly via an intramolecular mechanism. In the present work, because the suspensions of intact ghosts were mixed with vanadate under aerobic conditions, it is reasonable that those unsaturated fatty acids in the membrane will to an extent be peroxidized. The vanadyl resulting from the reduction of vanadate will disproportionate these peroxides to initiate the chain reaction, and then cause lipid peroxidation. The protein–protein interactions and the lipid peroxidation will reduce the flexibility of erythrocyte membrane and increase the rigidity. This is one of the reasons why the motion of lipid molecules in the bilayer decreases upon incubation with vanadate, as shown by the 5-NS spin-labeled ESR spectroscopy studies.

Conclusions

It was known that vanadate is transported rapidly into erythrocytes through an anion channel, in the same way as phosphate. Based on the results here reported, the transportation process of the vanadate ion is somewhat different from phosphate. It was shown by ^{51}V NMR studies that most of the monomeric vanadate (86%) binds to the anion binding sites of band 3. The binding is stronger than phosphate (Vetter *et al*. 1991), as the binding constant estimated from the fluorescence-quenching determinations was found to be $K_{S,20} = 4.3$ mM^{-1}. The vanadate binding causes changes in the structure and properties of erythrocyte membrane, but phosphate does not. Moreover, vanadate is different from phosphate in its susceptibility to reduction by sulfhydryl groups, leading to vanadyl formation, though only a small fraction of vanadate (3%) was reduced. Under aerobic conditions, the vanadyl is reoxidized to vanadate after a longer exposure to air. The reduction of vanadate is accompanied by oxidative cross-linking of membrane proteins and thus dampens their motility. Meanwhile, the reoxidation of vanadyl ions promotes the chain reactions of peroxidation and contributes to the increase in membrane rigidity. As shown by the time scale of each experiment in this work, the reaction between vanadate and erythro-

Table 2. Effect of vanadate on the physical state of erythrocyte membrane protein and lipid as monitored by the W/S ratio of MAL-6 and the order parameter of 5-NS

Concentration of vanadate (mM)	0	0.075	0.150	0.300	0.750	1.000
W/S(±SD)	4.12 ± 0.04	3.42 ± 0.06	3.21 ± 0.06	3.20 ± 0.07	3.07 ± 0.06	3.05 ± 0.05
Order parameter (±SD)	0.625 ± 0.005	0.631 ± 0.006	0.632 ± 0.006	0.640 ± 0.007	0.657 ± 0.007	0.658 ± 0.007

cyte ghosts is a time-dependent process. At first, the binding of vanadate to the membrane proteins takes place within several hours of incubation and causes the conformation changes of the membrane proteins. If the incubation time is prolonged, vanadate will react with sulfhydryl groups of membrane proteins, which results in the alteration of the structure of erythrocyte ghost membrane.

Acknowledgements

The National Natural Science Foundation of China and Zhongguancun United Analytical Center in Beijing are gratefully acknowledged for supporting this work.

References

Butterfield DA. 1982 Spin labelling in disease. In: Berliner LJ, Reaben J, eds. *Biological Magnetic Resonance*. New York: Plenum Press; **4**, 1–78.

Chasteen ND. 1981 Vanadyl (IV) EPR spin probes: inorganic and biochemical aspects. In: Berliner L, Reuben J, eds. *Biological Magnetic Resonance*. New York: Plenum Press; **3**, 53–119.

Chasteen ND. 1983 The biochemistry of vanadium. *Structure and Bonding* **53**, 105–138.

Chasteen ND, ed. 1990 *Vanadium in Biological systems: Physiology and Biochemistry*. The Netherlands: Kluwer Academic.

Chen BW, Bai YG, Wang K, Zhang BY. 1997 The effect of vanadium on protein and lipid peroxidation of erythrocyte membrane. *J Chinese Biochem*, **13**, 231–234

Chen LX, Lu JF, Wang K. 1992 The influence of bilirubin on fluidity and rotational correlation times of human erythrocyte membrane. *Cell Biol Internat Reports* **16**, 567–573.

Combeau C, Carlier MF. 1988 Probing the mechanism of ATP hydrolysis on F-actin using vanadate and the structural analogs of phosphate BeF_3^- and AlF_4^-. *J Biol Chem* **263**, 17429–17436.

Crans DC. 1994 Aqueous chemistry of labile oxovanadates: relevance to biological studies. *Comments Inorg Chem* **16**, 1–33.

Crans DC, Mahroof-Tahir M, Keramidas AD. 1995 Vanadium chemistry and biochemistry of relevance for use of vanadium compounds as antidiabetic agents. *Molecular and Cellular Biochemistry* **153**, 17–24.

Ellman GL. 1959 Tissue sulfhydryl groups. *Arch Biochem Biophys* **82**, 70–77.

Falke JJ, Chan SI. 1986 Molecular mechanisms of band 3 inhibitors. 1. Transport site inhibitors. *Biochemistry* **25**, 7888–7894.

Galanter WL, Labotka RJ. 1991 The binding of nitrate to the human anion exchange protein studied with ^{14}N nuclear magnetic resonance. *Biochim Biophys Acta* **1079**, 146–151.

Heinz A, Rubinson KA, Grantham JJ. 1982 The transport and accumulation of oxovanadium compounds in human erythrocytes *in vitro*. *J Lab Clin Med* **100**, 593–612.

Highsmith S, Barker D, Scales DJ. 1985 High-affinity and low-affinity vanadate binding to sarcoplasmic reticulum Ca^{2+}-ATPase labeled with fluorescein isothiocyanate. *Biochim Biophys Acta* **817**, 123–133.

Jay M, Stuart SM, McClain CJ, Palmieri DA, Butterfield DA. 1987 Alterations in lipid membrane fluidity and the physical state of cell-surface sialic acid in zinc-deficient rat erythrocyte ghosts. *Biochim Biophys Acta* **897**, 507–511.

Keller RJ, Sharma RP, Grover TA, Piette LH. 1988 Vanadium and lipid peroxidation: evidence for involvement of vanadyl and hydroxyl radical. *Arch Biochem Biophys* **265**, 524–533.

Lakowicz JR. 1983 *Principles of Fluorescence Spectroscopy*. New York: Plenum Press; 255–295.

Legrum W. 1986 The mode of reduction of vanadate(V) to oxovanadium(IV) by glutathione and cysteine. *Toxicology* **42**, 281–289.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951 Protein measurement with the folin phenol. *J Biol Chem* **193**, 265–275.

Macara IG, Kustin K, Cantley LC Jr. 1980 Glutathione reduces cytoplasmic vanadate mechanism and physiological implications. *Biochem Biophys Acta* **629**, 95–106.

Oltz EM, Bruening RC, Smith MJ, Kustin K, Nakanishi K. 1988 The tunichromes. A class of reducing blood pigments from sea squirts: isolation, structures, and vanadium chemistry. *J Am Chem Soc* **110**, 6162–6172.

Orvig C, Thompson KH, Battell M, McNeill JH. 1995 Vanadium compounds as insulin mimics. In: Sigel H, Sigel A, eds. *Metal Ions in Biological Systems*. New York: Marcel Dekker Inc.; **31**, 575–594.

Rehder D. 1991 The bioinorganic chemistry of vanadium. *Angew Chem Int Ed Engl* **30**, 148–167.

Stankiewicz PJ, Tracey AS. 1995 Stimulation of enzyme activity by oxovanadium complexes. In: Sigel H, Sigel A, eds. *Metal Ions in Biological Systems*. New York: Marcel Dekker Inc.; **31**, 249–285.

Stankiewicz PJ, Tracey AS, Crans DC. 1995 Inhibition of phosphate-metabolizing enzymes by oxovanadium(V) complexes. In: Sigel H, Sigel A, eds. *Metal Ions in Biological Systems*. New York: Marcel Dekker Inc.; **31**, 286–324.

Vetter IR, Hanssum H, Bäumert HG. 1991 Studies on the anion binding selectivity of sarcoplasmic reticulum membranes by ^{35}Cl-NMR. *Biochim Biophys Acta* **106**, 9–16.

Wyse J, Butterfield DA. 1989 Interaction of hemin with erythrocyte membranes: alterations in the physical state of the major sialoglycoprotein. *Biochim Biophys Acta* **979**, 121–126.

Zhang BY. 1996 *Studies on the Chemical Basis of the Similarities and Relativities between Oxovanadium (V,IV) Ions and Several Essential Ions in Biological Systems*. PhD Dissertation, Nanjing University, Nanjing, China.