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Direct vibrational structure of protein metal-binding sites from near-infrared Yb^{3+} vibronic side band spectroscopy

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ABSTRACT **Near-infrared Yb^{3+} vibronic side band (VSB) spectroscopy is used to obtain structural information of metal binding sites in metalloproteins. This technique provides a selective "IR-like" vibrational spectrum of those ligands chelated to the Yb^{3+} ion. VSB spectra of various model complexes of Yb^{3+} representing different ligand types were studied to provide references for the VSB spectra of Yb^{3+}-reconstituted metalloproteins. Ca^{2+} in the calcium-binding protein parvalbumin and Fe^{3+} in the iron-transporting protein transferrin were replaced with Yb^{3+}. The fluorescence of Yb^{3+} reconstituted into these two proteins exhibits weak VSBs whose energy shifts, with respect to the main $^2F_{5/2} \rightarrow {}^2F_{7/2}$ Yb^{3+} electronic transition, represent the vibrational frequencies of the Yb^{3+} ligands. The chemical nature of the ligands of the Yb^{3+} in these proteins, as deduced by the observed VSB frequencies, is entirely in agreement with their known crystal structures. For transferrin, replacement of the $^{12}CO_3^{2-}$ metal counterion with $^{13}CO_3^{2-}$ yielded the expected isotopic shift for the VSBs corresponding to the carbonate vibrational modes. This technique demonstrates enormous potential in elucidating the localized structure of metal binding sites in proteins.**

Many enzymes bind metals at catalytic or functional sites. The structural elucidation of these sites and the chemical nature of the metals' ligands are imperative if we are to understand how these enzymes function. For calcium-binding proteins, the structure of the binding site is particularly difficult to probe since Ca^{2+} is "spectroscopically silent" by most methods. Metal substitution in metalloproteins is, thus, useful to characterize the metal binding site and the function of proteins. Trivalent lanthanides are well known for their specificity in replacing calcium and other metals (1, 2), and several techniques using lanthanides have been extensively used, including fluorescence lifetime measurements, excitation transfer measurements, EPR, and NMR (2). These techniques, however, do not provide direct information concerning the chemical nature of the metal ligands.

Vibrational spectroscopy is a valuable tool in determining molecular structure and interactions. There are specific problems and difficulties in obtaining direct vibrational information on amino acid residues acting as ligands to metals in metalloproteins. One of the major difficulties is site specificity. If one wishes to obtain a vibrational IR absorption spectrum of the metal ligands in a protein, a difference technique with an appropriate differential trigger needs to be employed. The same holds true for a vibrational Raman spectrum. For a resonance Raman experiment, selectively obtaining a vibrational spectrum in the metal site will rely on exciting in resonance with a ligand-to-metal charge transfer band that usually exhibits a low extinction coefficient, thus minimizing the resonance enhancement of the ligand spectra. Of course, if the protein possesses many chromophores that are near or overlapping with this charge transfer absorption band, then the weak resonance Raman spectra of the metal ligand will most likely be masked by the overwhelming (pre)resonant Raman spectrum of the chromophore. We propose, as a solution to these problems, the technique of fluorescence vibronic side band (VSB) spectroscopy.

We have reported (3) the feasibility of using near-IR (NIR) VSB spectroscopy to directly obtain the vibrational structure of ligands in complexes of the lanthanide Yb^{3+} (3). This technique is based on the approach used by Macgregor and coworkers (4, 5) for the lanthanide Gd^{3+}, which fluoresces in the UV, but is complicated by protein fluorescence in this same spectral region. For Yb^{3+}, this problem is eliminated because its fluorescence is excited in the NIR, which also reduces the probability of photodegradation and actinicity in chromophore-containing proteins. The VBSs observed in the fluorescence spectrum of Ln^{3+} complexes are shifted to longer wavelengths with respect to the main $4f$–$4f$ (0–0) electronic transition of the lanthanide and arise from dipole–dipole coupling of the electronic transition (Yb^{3+}: $^2F_{5/2} \rightarrow {}^2F_{7/2}$) and the IR-active vibrational oscillators of the chelates; the shifts in energy (in cm^{-1}) correspond to the vibrational frequencies of the chelate oscillators and thus provide an "IR-like" vibrational spectrum. Since the dipole–dipole coupling with the lanthanide is expected to be greatest with the vibrational oscillators nearest to the lanthanide, the obtained IR-like spectrum corresponds to the ligands in the immediate coordination sphere of the lanthanide.

In this article, we report the VSB spectra of various Yb^{3+} complexes compared to their respective IR absorption spectra. As well, we report the VSB spectra of Yb^{3+} (*i*) in the Ca^{2+}-binding site of rabbit muscle parvalbumin (RMPA) and (*ii*) in the Fe^{3+}-binding site of the iron transport protein transferrin. By using these well-characterized metal binding sites, we have been able to demonstrate the ability of this technique to deduce ligand types and, for RMPA, to deduce the ratio of monodentate to bidentate carboxylate ligands bound to the Yb^{3+}, in agreement with the known crystal structure of Yb^{3+}-reconstituted parvalbumin (6).

MATERIALS AND METHODS

Apotransferrin (Sigma) was used without further purification and was dissolved at 50 μM in 10 ml of 25 mM Tris·HCl, pH 8.5/10 mM Na_2CO_3 as described (7). Protein concentration was measured by UV absorption of the solution by using the extinction coefficient $E^{1\%,\,1cm}_{280nm}$ of 11.2 (7). Yb^{3+} (as $YbCl_3 \cdot 6H_2O$, Aldrich) was added to this solution at a ratio of 10 Yb^{3+} per 1 apotransferrin. The mixture was stirred at room

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Abbreviations: VSB, vibronic side band; NIR, near-infrared; RMPA, rabbit muscle parvalbumin; DPA, dipicolinic acid; Im, imidazole; YbTHD, tris(2,2,6,6-tetramethyl-3,5-heptanodionato) ytterbium; L_2, 2-formyl-4-methyl-6-[*N*-(2-pyridylethyl)formimidoyl]phenol; EHPG, ethylenediamine di(*o*-hydroxyphenylacetic acid); ECR, eriochrome cyanine R; a.u., arbitrary unit(s).

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temperature for 30 min and then dialyzed for two 2-h periods at 5°C against 1 liter of 25 mM Tris·HCl, pH 8.5/10 mM Na_2CO_3. The sample was then concentrated to 1 ml with a microconcentrator (Centricon-10, Amicon). H_2O was exchanged with ^2H_2O by resuspending the concentrated protein twice in 10 ml of Tris carbonate buffer prepared in ^2H_2O at p^{2}H 8.5. Finally, the protein solution was lyophilized with a SpeedVac concentrator (Savant) and stored at −80°C until it was used. The protein containing $^{13}CO_3^{2-}$ or SO_4^{2-} was prepared in a similar way, using 50 mM $Na_2{}^{13}CO_3$ (Eurisotope, Saclay, France) or 50 mM Na_2SO_4, respectively, with 25 mM Tris, at p^{2}H 8.5. All buffers were first purged with argon prior to use, but the presence of a residual amount of $^{12}CO_3^{2-}$ cannot be excluded. The binding of 2 Yb^{3+} per dimer was confirmed by graphite furnace atomic absorption (Perkin–Elmer HGA 300-2380 spectrophotometer) and by the characteristic changes in the protein UV absorption spectrum (measured using a Cary 2300 spectrophotometer) (8).

RMPA (Sigma) was dissolved in ^2H_2O containing 150 mM NaCl (Merck, Suprapur)/15 mM Pipes buffered at p^{2}H 7.2. Yb^{3+} was then added to the solution with a ratio of 2 Yb^{3+} per dimer. The solution was stirred for 15 min at room temperature and then lyophilized.

NIR Yb^{3+} fluorescence was excited with a continuous wave Ti/sapphire laser (Spectra Physics model 3900 S) and the spectra were recorded using a modified Bruker IFS 66 Fourier transform IR spectrometer with a Bruker FRA 106 Raman module as described (3) but with the following modifications: the excitation wavelength was variable between 970 and 940 nm and stray light was rejected with custom-made holographic notch filters (Kaiser Optical Systems, Ann Arbor, MI). Samples were either prepared in ^2H_2O to avoid the absorption of the exciting light by H_2O or used as a powder and spectra were all recorded at 15 K, by using a helium gas-flow cryostat (SMC, Orly, France). All Raman contributions were identified by changing the excitation wavelength by ≈30 cm^{-1} and recording the VSB spectrum of the same sample. The frequency of the principal fluorescence band was determined using a Jobin-Yvon U1000 double monochromator equipped with a charge-coupled device camera and an exciting wavelength of 894 nm. Depending on the complex or protein, the values found varied between 974 nm and 979 nm (data not shown). VSB frequencies are then calculated with these values. Attenuated total reflection Fourier transform IR absorption spectra were recorded at room temperature on a Bruker IFS 66 spectrophotometer equipped with a 45° ZnSe attenuated total reflection attachment.

The Yb^{3+} complexes were prepared mixing Yb^{3+} stock solution with the various ligands (see Table 1). Tris(2,2,6,6-tetramethyl-3,5-heptanodionato)ytterbium (YbTHD) was purchased (Aldrich), YbTHD imidazole (Im) and YbTHD 4-methyl imidazole (4MeIm) were prepared as in ref. 9, and Yb–eriochrome cyanine R (ECR) was as in ref. 10.

RESULTS AND DISCUSSION

As lanthanides and calcium are known to have a strong affinity for oxygen ligands, we studied Yb^{3+} model complexes containing carboxylate (COO^-) groups. For these chemical groups, intense IR vibrational bands are expected at ≈1400 cm^{-1} and ≈1600 cm^{-1}, corresponding to the symmetric and antisymmetric COO^- vibration stretching modes, respectively (11). Fig. 1 shows the fluorescence spectrum of the Yb^{3+}–dipicolinic acid (DPA) complex. The intense band at 977 nm corresponds to the main $^2F_{5/2} \rightarrow {}^2F_{7/2}$ electronic transition; VSB frequency shifts are determined relative to this transition. Spectrum a in the inset of Fig. 1 is the VSB spectrum of YbDPA. Spectrum b is the IR absorption spectrum of YbDPA. Spectrum c is the IR absorption spectrum of

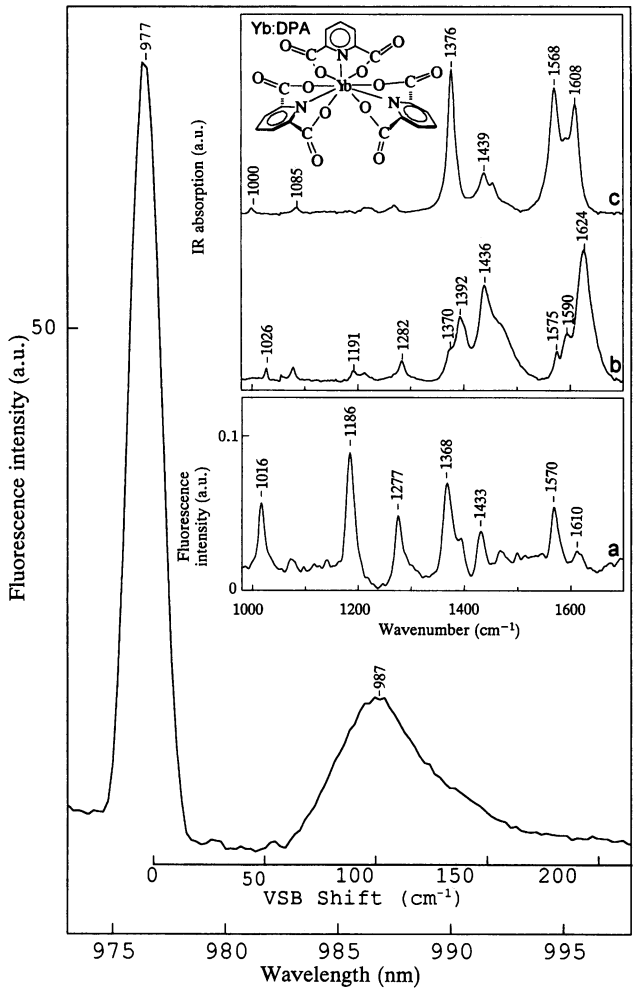

FIG. 1. Fluorescence spectrum of Yb^{3+} complexed with DPA and excited at 894 nm. The fluorescence spectrum corresponds to one scan at 15 K. (*Insets*) Spectra: a, VSB spectrum of YbDPA, excited at 941 nm (light intensity, 20 mW), average of 2000 scans; b, IR spectrum of YbDPA, average of 50 scans; c, IR spectrum of DPA, average of 50 scans. a.u., Arbitrary unit(s).

the DPA alone in its deprotonated state (p^{2}H 10). The bands that are shifted in spectrum b compared to spectrum c correspond to the vibrations of the ligand functional groups implicated in the binding of Yb^{3+}. The COO^- symmetric stretch varies from 1376 cm^{-1} in spectrum c to 1370 cm^{-1} in spectrum b. The COO^- antisymmetric stretch varies from 1568 cm^{-1} in spectrum c to 1575 cm^{-1} in spectrum b. The feature at 1000 cm^{-1} in spectrum c, which is shifted to 1026 cm^{-1} in spectrum b, corresponds to a pyridine mode (12, 13). Based on these observed shifts, one would expect that ligation of Yb^{3+} by DPA occurs via the oxygens of the carboxylic groups and the nitrogen of the pyridine ring, which is in agreement with the crystal structure of YbDPA (14). Spectra a and b of Fig. 1 *Insets* show that the VSB frequencies match gratifyingly well with the corresponding IR frequencies and they are attributed to COO^- and pyridine vibrations (12, 13). These assignments are also consistent with the crystal structure of YbDPA complex (14), since in VSB spectra the frequencies of the ligands are the main features. Since the IR and VSB spectra result from two physical phenomena and are governed by different selection rules (15), it is expected that the relative intensities of peaks in the various spectra are not identical (i.e., compare the intense broad IR band at 1624 cm^{-1} in Fig. 1, spectrum b,

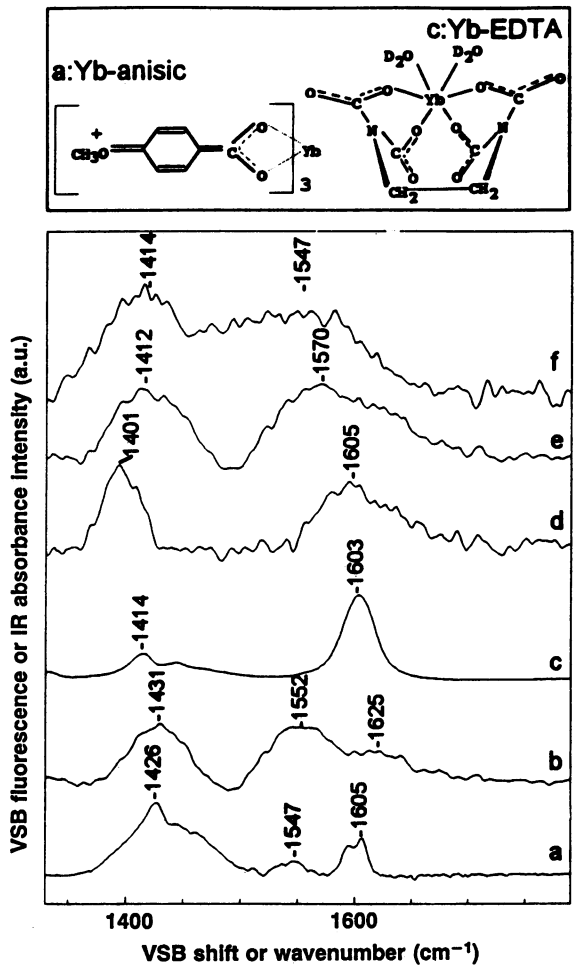

FIG. 2. Comparison of the IR and VSB frequencies of the COO^- stretches of carboxylic groups. Spectra: a, IR spectrum of YbAnisic acid, average of 50 scans; b, VSB spectrum of YbAnisic acid, excited at 945 nm (light intensity, 10 mW), average of 4000 scans; c, IR spectrum of YbEDTA, average of 50 scans; d, VSB spectrum of YbEDTA, excited at 942 nm (light intensity, 120 mW), average of 2000 scans; e, 3b + 5d calculated addition spectrum of spectra b and d with weighting factors 3 and 5, respectively; f, VSB spectrum of RMPA, excited at 940 nm (light intensity, 60 mW), average of 6000 scans. D, ^2H.

with the relatively less-intense narrower VSB band at 1610 cm^{-1}).

Lanthanides can bind carboxylic acids in a monodentate or bidentate manner. Fig. 2 shows VSB spectra (spectra b and d) and IR spectra (spectra a and c) obtained with the complexes YbAnisic acid and YbEDTA, respectively. For YbEDTA, the carboxylic groups of EDTA are coordinated in a monodentate manner, according to its crystal structure (16), whereas for YbAnisic acid, the binding mode of the carboxylic group is bidentate (17). The observed difference, $\Delta\nu$, between the symmetric and antisymmetric vibrations is $\approx$200 cm^{-1} for YbEDTA (Fig. 2, spectrum d). This difference is greater than for a bidentate COO^- ligand, as it is verified in the YbAnisic acid complex where $\Delta\nu$ = 121 cm^{-1} (Fig. 2, spectrum b). This shows that the nature of the YbCOO ligation can be determined by the frequencies of the stretching vibrations in VSB spectra, which can be used to analyze metal binding sites of proteins.

This analysis was applied to RMPA as seen in Fig. 2 where spectrum f is the VSB spectrum of the Yb^{3+}-reconstituted RMPA. From the crystal structure of RMPA where Yb^{3+} replaces Ca^{2+} (6), we expect that Yb^{3+} is ligated to three bidentate and five monodentate carboxylic groups. Spectrum e results from the average in the same ratio of the VSB spectrum of the YbAnisic acid and YbEDTA complexes and is in agreement with spectrum f. In addition to the COO^- bands, RMPA spectrum exhibits another band at 1130 cm^{-1} (data not shown), which can arise from the C–O bond vibration of the serine ligand. As with Ca^{2+}, the bonding nature of lanthanides with ligands is known to be more ionic than covalent (2). This would explain why the frequency of the symmetric COO^- stretch is found below 1600 cm^{-1}.

Fig. 3 shows spectra of other different complexes studied, representative of other ligand functional groups. The observed VSB frequencies and their tentative assignments, based on the literature, are summarized in Table 1. In the complexes in which ^2H_2O is one of the ligands of Yb^{3+}, a

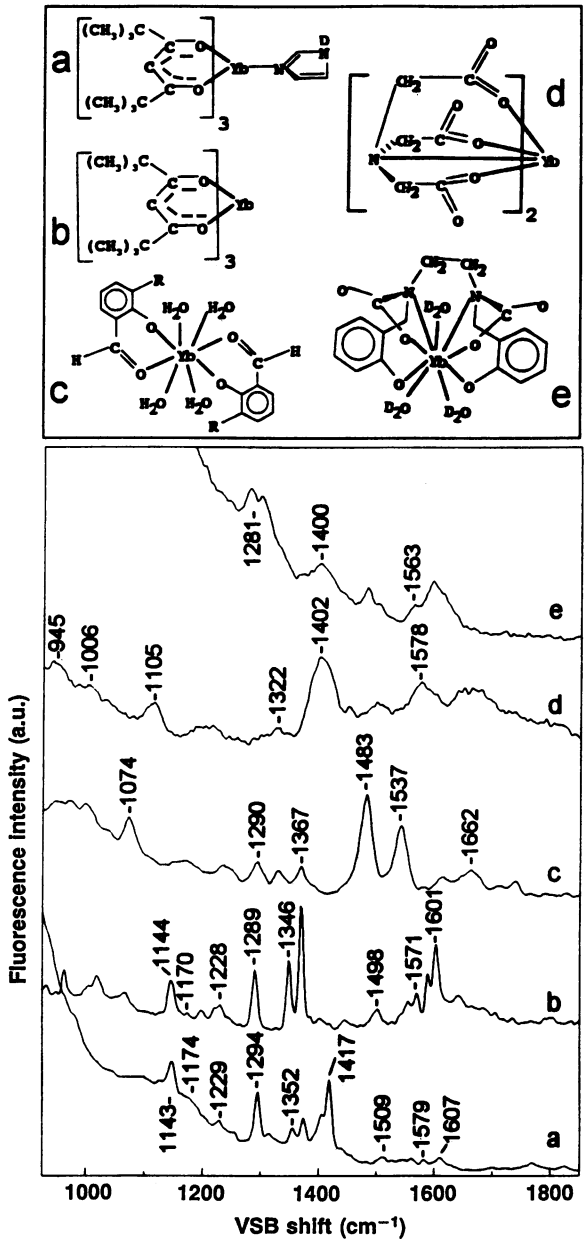

FIG. 3. VSB spectra of model compounds. Spectra: a, YbTHDIm as a powder, excited at 945 nm (light intensity, 20 mW), average of 4000 scans; b, YbTHD as a powder, excited at 955 nm (light intensity, 10 mW), average of 2000 scans; c, YbL_2 as a powder, excited at 940 nm (light intensity, 10 mW), average of 4000 scans [R = $CN(CH_2)_2$ pyridine]; d, Yb–Nitrilotriacetic acid, excited at 942 nm (light intensity, 25 mW), average of 2000 scans; e, YbEHPG, excited at 940 nm (light intensity, 10 mW), average of 8000 scans. D, ^2H.

Table 1. Observed VSB frequencies of various chemical groups compared to corresponding IR bands and their tentative assignments

	Frequency																
Compound	Ring C-C	ν aC=O	C=C	NH bend	C=C=C	Ring br	ν sC=O	Ring str	Ring C-C	CO_2	C-C	C-C-O	C-O	C-H	Ring def.	CN	Ref(s).
YbEDTA		1605 (1603)					1401 (1414)									1118 (1105)	16*, 18, 19
YbNTA		1578 (1602)					1402 (1404)			1322 (1322) 1006 (1015) 945 (944)						1105 (1125)	20
YbDPA	1610 (1624)	1570 (1575)				1433 1436	1368 (1370)		1277 (1282)						1186 (1191)	1016 (1026)	12, 14*
YbAnisic	1625 (1605)	1552 (1547)					1431 (1426)										11
YbEHPG	1597 (1590)	1563 (1573)				1483 (1478)	1400 (1401)						1281 (1288)				21, 22*
YbECR		1626 ket (1618) 1572 (1568)				1471 (1473)	1417 (1427)				1327 (1323)		1284 (1291)		1023 (1033)		10
YbL_2		1662 al (1664)	1537 (1533)			1483 (1479)			1367 (1366)				1290 (1291)		1074 (1063)		23, 24*
YbTHD		1601 (1592)	1571 (1574)		1498 (1506)		1369 (1355)				1289 (1287)	1228 (1225)	1170 (1175)	1144 (1140)			9*, 25
YbTHD-Im		1607 (1592)	1579 (1574)	1559	1509 (1506)		1352 (1355)				1294 (1287)	1229 (1225)	1174 (1175)	1143 (1140)			9
YbTHD-4MeIm		1601	1553		1487		1375				1270		1162	1123			9

Frequencies in parentheses represent IR frequencies. al, Aldehyde; ket, ketone; br, breathing; str, stretching; def, deformation. Complexes of ytterbium were prepared by mixing 10 mM $YbCl_3 \cdot 6H_2O$ with 10 mM EDTA (p^2H 6.1), 10 mM nitrilotriacetic acid (NTA) (p^2H 9), 50 mM DPA (p^2H 5.2), 50 mM anisic acid (p^2H 6.5), 10 mM EHPG (p^2H 10.5), 100 mM $Na_2{}^{12}CO_3$ (p^2H 6.1), 30 mM $Na_2{}^{13}CO_3$ (p^2H 6.1) in ^2H_2O, and buffered with ^2HCl or NaO^2H. YbTHDIm, YbTHD, and 4MeIm were prepared as in ref. 9 and YbECR was as in ref. 10. YbL_2 (22) was the kind gift of O. Kahn (24).

*Description of the structure of the complexes used.

vibration is observed between 2500 and 2600 cm^{-1}, which is consistent with an OD stretching mode (see also ref. 4). An OD bending mode is also observed at 1213 cm^{-1} when Yb^{3+} is ligated only by ^2H_2O (data not shown).

VSB intensities are expected to depend on the precise location and orientation of the ligands with respect to Yb^{3+} (26). The IR spectrum of YbTHDIm is the exact superposition of the IR spectrum of YbTHD and the IR spectrum of YbIm (data not shown but see also ref. 9). In contrast, the VSB spectrum of YbTHDIm (Fig. 3, spectrum a) does not represent the superposition of VSB spectra of YbTHD (Fig. 3, spectrum b) and of YbIm (spectrum not shown) even when excited at the same wavelength. The dramatic difference in relative intensities is most likely due to different ligand orientations for these two complexes, which differ by one extra Im ligand. This effect is also seen when comparing the VSB spectra of YbDPA in solution and YbDPA crystals (data not shown). In this case, this may be due to the different symmetries of the two complexes or the effect of the crystal field in altering the activity of certain ligand vibrations (26). A conspicuous new band appears in the VSB spectrum when an Im ligand is added (compare spectra a and b in Fig. 3). The band at 1417 cm^{-1} is not present in IR spectra of Im but an intense band at ≈1410 cm^{-1} is observed in Raman spectra of N-deuterated imidazolium ring of histidine (27). Since in some cases Raman-active vibrational modes can be active in rare earth VSB spectra (15), the band at 1417 cm^{-1} in the VSB spectrum of YbTHDIm complex is a candidate for an Im VSB feature.

Transferrin is a dimeric protein and each 40-kDa subunit contains an iron binding site (28, 29). In this protein, the iron is ligated by two tyrosines, one histidine, one monodentate aspartic acid, and one bidentate carbonate. Fig. 4 shows spectra of Yb^{3+}-reconstituted transferrin with three anions bound to Yb^{3+}: $^{12}CO_3^{2-}$ (spectrum b), $^{13}CO_3^{2-}$ (spectrum a), and SO_4^{2-} (spectrum c). The carbonate vibrations are observed at 1548 cm^{-1} and 1438 cm^{-1} with $^{12}CO_3^{2-}$ (spectrum b) and at 1514 cm^{-1} and 1412 cm^{-1} for $^{13}CO_3^{2-}$ (spectrum a), based on the spectra obtained with $Yb(^{13}CO_3)_3$ and $Yb(^{12}CO_3)_3$ (Fig. 4 *Inset*). The feature at 1132 cm^{-1} in spectrum b is attributed to a carbonate stretching mode (30). Both in $Yb(CO_3)_3$ complex spectrum (data not shown) and in transferrin spectrum, no shift is observed for this band with ^{13}C substitution. This could be due to a low sensitivity of the COO^- symmetric stretch to isotopic exchange on the carbon, as this vibration mainly implicates the oxygen of the CO_3^{2-}. By exchanging carbonate with sulfate, VSB features that were first masked by carbonate bands can now be observed. Features attributed to SO_4^{2-} binding appear between 997 cm^{-1} and 1090 cm^{-1} (26, 31). Bands also appear at 1344 cm^{-1} and 1369 cm^{-1}. These frequencies agree with those expected for tyrosine (32) or a phenolic ligand (23). Indeed, in Yb–2-formyl-4-methyl-6-[*N*-(2-pyridylethyl)formimidoyl]phenol (L_2) (Fig. 3, spectrum c), Yb–ethylenediamine di(*o*-hydroxyphenylacetic acid) (EHPG) (Fig. 3, spectrum e), and YbECR (Table 1) complexes, which all contain a phenolic ligand, the band at ≈1263 cm^{-1} can be assigned to a C–O stretch (33) and the feature at 1369 cm^{-1} can be assigned to a C–C stretch of the phenol ring (23). Weak bands at 1560 cm^{-1} and 1443 cm^{-1} are observed and may be consistent with the COO^- antisymmetric and symmetric bands of the carboxylic group of the aspartic ligand. The difference in energy between these frequencies is ≈117 cm^{-1},

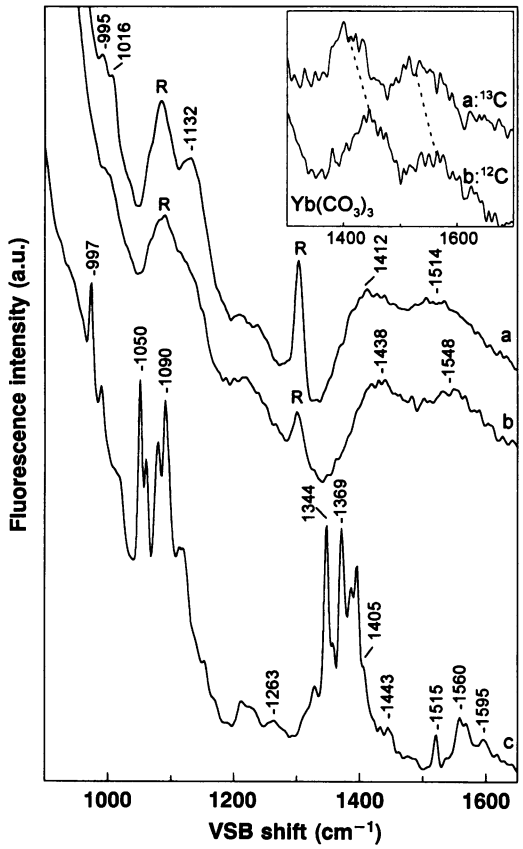

FIG. 4. VSB spectra of Yb^{3+} transferrin with three anions: Spectra: a, $^{13}CO_3^{2-}$-reconstituted transferrin, excited at 940 nm (light intensity, 55 mW), average of 8000 scans; b, $^{12}CO_3^{2-}$-reconstituted, excited at 940 nm (light intensity, 80 mW), average of 4000 scans; c, SO_4^{2-}, excited at 940 nm (light intensity, 100 mW), average of 6000 scans. (*Inset*) VSB spectra of $Yb(CO_3)_3$. Spectra: a, $Yb(^{13}CO_3)_3$, excited at 940 nm (light intensity, 45 mW), average of 6000 scans; b, $Yb(^{12}CO_3)_3$, excited at 940 nm (light intensity, 10 mW), average of 3000 scans. Raman bands are identified by R in the spectra.

consistent with the COO^- antisymmetric and symmetric bands of the carboxylic group of the aspartic ligand. The difference in energy between these frequencies is $\approx 117\ cm^{-1}$, and as it has been shown above, it should correspond to a bidentate carboxylic group. This means that monodentate binding of iron by the carboxylic group of aspartic acid could be bidentate with Yb^{3+}. This effect was already observed with RMPA, where replacement of Ca^{2+} by Yb^{3+} resulted in a monodentate to bidentate binding by Asp-92 (6). This could be expected since lanthanides can accommodate more than six ligands, although iron usually coordinates no more than six ligands.

Based on the IR-active vibrational modes of histidine, we have not been able to unambiguously identify VSB bands arising from the histidine ligand in transferrin. In general, the IR absorption extinction coefficient of Im is much smaller than that of a carbonyl oxygen-containing ligand (32), and thus the histidine vibrational dipole may not couple as effectively to the Yb^{3+} electric dipole. However, the shoulder seen at 1405 cm^{-1} in the transferrin VSB spectrum could correspond to 1417 cm^{-1} band seen in the VSB spectrum of the YbTHDIm complex.

NIR VSB spectroscopy is a powerful technique for qualitative study, as is shown with YbECR in which four kinds of oxygen ligands were detected (Table 1), and since (*i*) it avoids protein fluorescence, actinicity, and photodegradation; (*ii*) detection in the NIR allows a greater resolution than in the UV; and (*iii*) a very sensitive Fourier transform Raman detector can be used.

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