Binding of Vitamin B_{12} and B_{12a} to an Antibody and to Haptocorrin Probed by Enantioselective Quenching of Tb(pyridine-2,6-dicarboxylate)$_3^{3-}$ Luminescence

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Chiral discrimination in the quenching of the luminescence from dissymmetric lanthanide complexes such as Tb(III) tris-(pyridine-2,6-dicarboxylate dianion = DPA) by chiral transition-metal compounds provides a clear example of molecular recognition between reacting species. Such studies have involved metal tris chelates,[1] Cr or Rh nucleotide complexes,[2] ferri cytochrome *c*,[3] blue copper proteins, and vitamin B_{12} derivatives as quenchers.[4] The luminescence quenching by B_{12} and B_{12a} has been ascribed to energy transfer from the luminophore to the corrin chromophore and it has been shown[4] that it occurs at short distance in a specific, hydrogen-bonded, complex formed by diffusional encounter of an excited lanthanide species and the cobalamins. Here we report that complexation of the corrinoids with $B_{12(a)}$−binding proteins, which affects the accessibility of the corrinoid in the $B_{12(a)}$−protein complex toward the luminophore, is reflected in the enantioselective-quenching behavior. The latter property therefore provides information on the binding site(s) of the proteins for B_{12}; the chiral lanthanide complex acts as a probe for the complexation of the two biomolecules.

In aqueous solution at room temperature, Tb(DPA)$_3^{3-}$ (abbreviated, Tb) occurs as a racemic mixture of the Λ and Δ enantiomers. Interconversion rate is fast on the laboratory time scale, but slow as compared to the lifetime of the excited state (Tb*, 2.1 ms). Addition of small quantities of the corrinoids causes a shortening of the lifetime of the Λ-Tb* and Δ-Tb* species and, due to chiral discrimination in the dynamic quenching, the Λ-luminophore is quenched faster than its enantiomer.[4] This gives rise to a biexponential decay of the Tb* luminescence (with decay constants $k_1 = k_o + k_q^{\Delta}[Q]$ and $k_2 = k_o + k_q^{\Lambda}[Q]$, where k_o is the reciprocal lifetime in the absence of quencher and [Q] is the quencher concentration). It also leads to a development of circular polarization in the luminescence (CPL) after exciting the Tb species with a pulse of unpolarized light.[1c] Analysis of the luminescence intensity decay curve yields the pair of bimolecular quenching rate constants, k_q^{Δ} and k_q^{Λ}, from which one finds the absolute value of the degree of enantioselectivity in the quenching reaction, $E_q = (k_q^{\Delta} - k_q^{\Lambda})/(k_q^{\Delta} + k_q^{\Lambda})$. Analysis of the time-dependent CPL signal yields a value for $(k_q^{\Delta} - k_q^{\Lambda})$, which can be compared to the same quantity obtained from the (less time consuming) luminescence decay technique and allows for an important independent check on the internal consistency of the two analyses.[1c]

Anti-B_{12} antibody and haptocorrin (HC), a protein also known as non intrinsic factor or R-protein,[5] bind B_{12} and B_{12a} very strongly,[6−8] therefore in our experiments the dissociation of the protein−B_{12} complexes may be neglected.

Typical results for the complexation of the monoclonal antibody with B_{12} and B_{12a} are shown in Figure 1. Addition of anti-B_{12} to a solution containing 0.2 mM Tb(DPA)$_3^{3-}$ and 3 μM B_{12} results in an initial increase of the two diastereomeric rate constants, k_q^{Λ} and k_q^{Δ}. When the ratio of the *total* concentrations of antibody and B_{12} ([anti-B_{12}]/[B_{12}]) is near 0.5, a point of inflection occurs. Upon further increase of immunoglobulin concentration, the rate constants show an overall decrease. It is known that the antibody can bind two B_{12} molecules,[9] and this explains the changes in the titration curves near [anti-B_{12}]/[B_{12}] = 0.5. We have verified that in the absence of vitamin B_{12} the proteins do not quench Tb(DPA)$_3^{3-}$ emission. At each antibody concentration studied, the quenching rate constants have been determined by fitting a biexponential function with equal amplitudes to the emission decay trace.[1c] Time-resolved CPL measurements at [anti-B_{12}]/[cobalamin] = 0 and ∼0.5 yield results consistent with that from the luminescence decay experiments.

While for the bare B_{12} only a very small dependence of the quenching rate on ionic strength (I) is observed,[4] consistent with B_{12} being a neutral species, addition of NaCl to a sample with [anti-B_{12}]/[B_{12}] ≈ 0.5 significantly reduces the quenching rates of Tb*. This indicates that the increased quenching ability of the antibody-B_{12} complex is, at least partly, due to an attractive electrostatic interaction between Tb(DPA)$_3^{3-}$ and the complex. In turn, this shows that the antibody has a positive charge density near each of the two (equivalent[9]) B_{12} binding sites, implying the presence of lysine, aginine, or histidine residues in the binding region of the antibody. We explain the slow decrease of the rate constants in the region [anti-B_{12}]/[B_{12}] > 0.5 by the increase of ionic strength due to addition of antibody solution.

The enantioselectivity (E_q) in the quenching of Tb* by B_{12} amounts to -0.22 ± 0.01, see Figure 1. Upon addition of antibody, it decreases and appears to reach a plateau value of about $E_q = -0.04 \pm 0.015$, which we interpret as the chiral discrimination when the antibody−B_{12} complex acts as the quencher.

With B_{12a} (lower part of Figure 1) the behavior of E_q with increasing antibody concentration is very similar to that with B_{12} but the quenching rates behave more complex. First, a sharp decrease is observed, followed by an increase until, near [anti-B_{12}]/[B_{12a}] = 0.45, an inflection point is reached; finally, the rates slowly decrease. The initial decrease, which is the most striking difference in comparison with B_{12a}, deserves further study.[10,11]

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(5) Monoclonal anti-vitamin B_{12} (mouse ascites fluid) clone CD-29 (Lot 061H4824) was obtained from Sigma Chemicals. Haptocorrin was purchased from Sigma Chemicals as well where it is available under the name non intrinsic factor from porcine gastric mucosa (Lot 33H9522). Concentrations of these B_{12}-binding proteins were calculated from data supplied by the manufacturer. In all measurements, Tb(DPA)$_3^{3-}$ luminescence was monitored at 542.8 nm with a spectral band width of 2 nm. All data reported here pertain to aqueous solutions at room temperature.

(6) The association constant K_a of HC and B_{12a} is 5×10^{16} M^{-1} (ref 7); K_a values of the antibody−antigen complexes have not been reported, but they are expected to be on the order of 10^9 M^{-1} (ref 8).

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(10) At pH 7.2, the predominant species (70%) in a solution of B_{12a} (p*K*a 7.6 ± 0.2, ref 11) is the cation H_2OCbl^+, which is expected to give rise to a stronger I-dependence than the neutral B_{12}. Another possible explanation is that azide anions, added to the commercially available antibody solution as a preservative, replace the axial β water ligand of Co in the cobalamin. Azide does not substitute the cyanide ligand in B_{12} (ref 11).

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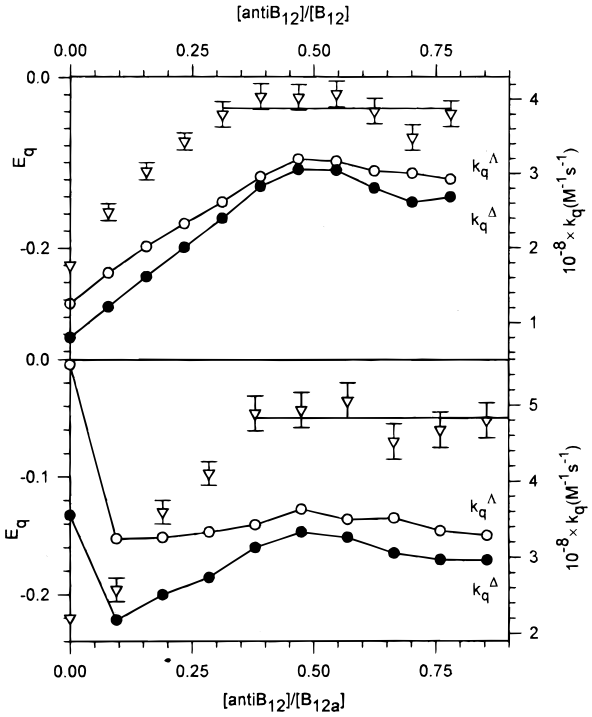

Figure 1. Effect of anti-B_{12} antibody on the quenching of $Tb(DPA)_3^{3-}$ luminescence by B_{12} (upper half) and B_{12a} (lower half). E_q values (triangles, vertical bars denote the standard error) and values of k_q^{Λ} (open circles) and k_q^{Δ} (filled circles) plotted against the ratio of the total cobalamin and antibody concentration. Sample conditions: $[Tb(DPA)_3^{3-}] = 0.2$ mM, 10 mM Hepes buffer, pH 7.2, $[B_{12(a)}] = 3\ \mu$M.

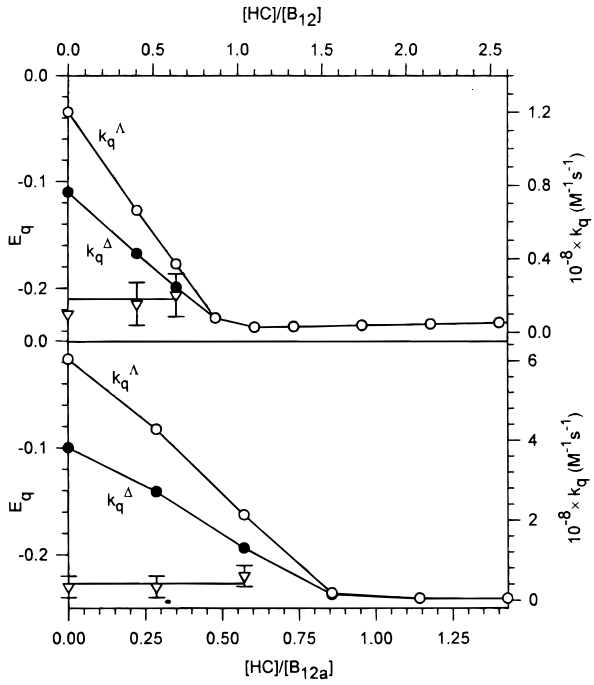

Figure 2. Effect of haptocorrin on the quenching of $Tb(DPA)_3^{3-}$ luminescence by B_{12} and B_{12a}. Symbols and sample conditions are as in Figure 1, except for $[B_{12}] = 9\ \mu$M and $[B_{12a}] = 7\ \mu$M.

The observation that quenching by the protein−cobalamin complex is very efficient, combined with the notion that the energy transfer rate strongly decreases with Tb*−quencher distance, says that the B_{12} and B_{12a} molecules are not shielded by the antibody from the solvent but rather are bound at the outside of the protein, still accessible to the luminophores in the solution. The change in E_q upon complexation with the immunoglobulin gives further information on the structure of the antibody−cobalamin complex. We elsewhere report[4] that the enantioselectivity in the quenching by free B_{12} and B_{12a} ($E_q \approx -0.22$) reflects differences in the strength of binding of Λ- and Δ-Tb to, most likely, the amide group on the a and g amide side chains of the cobalamins (and concomitant differences in the energy transfer rate in the two diastereomeric complexes). The different value of E_q for the vitamin-antibody complexes (∼-0.04), shows that the energy transfer no longer involves the same site on the cobalamin. This then, in turn, indicates that the a and/or g chain are involved in the binding of the cobalamin to the antibody.

Figure 2 shows the effect of haptocorrin on the quenching process with B_{12} and B_{12a}. In contrast to the case of anti-B_{12}, an almost linear decrease of the quenching rate constants upon addition of HC is observed until an equivalence point is reached near $[HC]/[B_{12}] = 0.9$ and $[HC]/[B_{12a}] = 0.85$, indicating the binding of one corrinoid per protein molecule. The HC/cobalamin complexes are very inept quenchers; from the data in Figure 2, their quenching rates are estimated to be $\sim 3 \times 10^6\ M^{-1}\ s^{-1}$, which is more than 1 ($B_{12}$) and 2 ($B_{12a}$) orders of magnitude smaller than that of the free vitamins.

For samples with $[HC]/[B_{12}]$ or $[HC]/[B_{12a}] > 1$, raising the ionic strength to 0.3 M does not measurably increase the quenching rate. Due to the smallness of the quenching rate constants, E_q values for the HC−cobalamin complexes could not be determined. The E_q data in the region $0 <$ [HC]/[cobalamin] < 0.6 indicate that the enantioselectivity is constant, which is consistent with the complexes being inefficient quenchers. This, in turn, points to a structure in which the cobalamin is shielded from the Tb* species in the solution by the haptocorrin. The shielding might be due to electrostatic repulsion between negatively charged groups in the vicinity of the binding site of the protein, but the observed insensitivity of the quenching ability of the protein complex toward salt concentration argues against this explanation because counter ions are expected to attenuate this repulsion. Encapsulation of the cobalamin by the protein could also prohibit contact between lanthanide complex and quencher. The scarce information available on the structure of the HC−B_{12} complex is that cobalamines bind to (chicken serum) haptocorrin nucleotide loop down with the upper (β) axial ligand facing the solution and accessible for substitution.[12] There are indications that the cobalamin is held rigidly by the protein and that its benzimidazole ligand may be replaced by a ligand donated by the protein upon binding.[13] Of this structural information, the accessibility of the β axial ligand of Co(III) for substitution contrasts with the shielding of the cobalamin by HC from the Tb-chelate, which we propose.

In conclusion, the enantioselective-quenching $Tb(DPA)_3^{3-}$ luminescence yields structural information about the B_{12}−protein complexes. The B_{12} and B_{12a} molecules bind to the outside of the antibody near positively charged amino acid residues, and they are well accessible to ions in the solution. This binding probably blocks the amide groups of the a and/or g side chain in the corrinoids. In contrast, B_{12} or B_{12a} which binds 1:1 to haptocorrin, is shielded from negative ions in solution, suggesting that the cobalamin is "buried" in the protein or perhaps attached at a site with negatively charged residues in the neighborhood. The present results offer also an analytical prospect: quenching of $Tb(DPA)_3^{3-}$ emission can be used as a probe to determine the stoichiometric point of complexation reactions of corrinoids with proteins.

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