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HEME PROTEIN STRUCTURE AND DYNAMICS, STUDIED BY RESONANCE RAMAN SPECTROSCOPY

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Abstract - Resonance Raman spectroscopy provides a structure probe for the iron porphyrin complex of heme proteins. It is capable of monitoring the time evolution of the structure when carried out with pulsed laser excitation, and/or flow techniques. The prompt photoproduct of COHb(Hb = hemoglobin), produced and detected with 30 ps pulses from a synchronously pumped mode-locked dye laser showed a RR spectrum with porphyrin skeletal frequencies characteristic of an in-plane high-spin Fe^{II} heme. This result implies that photoexcitation is followed by intersystem crossing to a high-spin ligand-field state of COHb, which is dissociative with respect to CO: the Fe atom, however, is constrained from moving out of the heme plane to the normal deoxy-heme structure. The subsequent out-of-plane relaxation was monitored via the porphyrin skeletal frequencies in pulsed laser and flow experiments; its time constant was bracketed between 20 and 300 ns, much longer than expected for simple atomic displacements. Since X-ray crystallography has shown that deligation of Hb is accompanied by movement (~1Å) of the entire F helix to which the proximal imidazole ligand is attached, it can be inferred that the out-of-plane Fe relaxation requires a large scale protein conformation change, to which it is coupled. This coupled motion may be part of the molecular mechanism for the R → T transition, which occurs later. The O_2Hb photointermediate, produced and detected by higher intensity (5 nj) 30 ps YAG pulses showed a different RR spectrum, with substantially down-shifted skeletal frequencies, suggestive of an electronically excited state with π^* orbital occupation. This spectrum relaxes rapidly, to one similar to that of the COHb photoproduct. The iron-imidazole stretching mode has been located in the RR spectrum of R-state deoxyHb using flow techniques, and found to be symmetrical (nearly equivalent α and β chain contributions) and upshifted relative to T-state deoxyHb, as is observed in chemically modified Hb's. The R-T frequency difference represents an appreciable bond energy change, which may be due to molecular strain or to changes in H-bonding.

INTRODUCTION

The coordination chemistry of the heme group (1,2) underlies the O_2 binding activity of hemoglobin (Hb) and myoglobin (Mb). The ligand field of the porphyrin ring appears to be poised near the spin crossover energy of Fe^{II}, so that the number and strength of the axial ligands determines the spin-state, and the bond lengths, of the heme complex. In deoxyHb or deoxyMb, there is only one axial ligand, the proximal imidazole, and the heme contains high-spin Fe^{II}. The bonds are long and the Fe atom is located some 0.5Å above the porphyrin in plane, on the side of the proximal imidazole. When O_2, or CO or NO, binds, the resulting complex is low-spin, with short bonds, and the Fe atom is in the porphyrin plane. This profound electronic and structural change is linked to alterations in the globin tertiary structure, which, in the case of Hb, lead to the change in quaternary structure that is believed to be associated with cooperativity in O_2 binding (3,4,5).

Consequently the structure of the heme group, and the dynamics of its alterations, are central to an understanding of Hb function. Resonance Raman spectroscopy provides a sensitive monitor of heme structure (6,7,8). Laser excitation into the strong heme absorption bands in the visible and near-UV region produces selective enhancement of Raman bands associated with heme vibrational modes, whose frequencies and intensities are responsive to the heme structure. The systematics of these modes have been worked out in some detail, and the technique has been applied to a wide range of heme proteins. Increasingly it is being used in a time-resolved mode, to study the structures of kinetic transients.

RR SPECTRA AND HEME STRUCTURE

Porphyrin Skeletal Modes

Resonance enhancement is greatest for Raman bands associated with vibrational modes that resemble the geometric distortion of the molecule in the resonant excited state (9). Since the heme absorption spectrum is dominated by the π-π^* transition (10), the principal RR bands are associated with stretching vibrations of the π bonds in the porphyrin ring (6,7). Excitation in the very intense Soret (B) band (~400 nm) mainly enhances totally symmetric vibrations, via the normal Frank-Condon, A term scattering (11,12) mainly, while excitation in the weaker α and β (Q) bands (~500-600 nm) emphasizes the non-totally symmetric vibrations which are effective in mixing the Q and B bands, via the vibronic, B term mechanism (11,12). The effective D_{4h} symmetry of heme facilitates the use of polarization measurements to identify the symmetry class of the modes, including the anomalously polarized A_{2g} modes, which are only observed at resonance (13).

It has been found empirically (14-17) that the porphyrin skeletal modes above 1450 cm^{-1} decrease in frequency when the porphyrin core is expanded via interactions of the central metal ion and its axial ligands. The slopes of the linear correlations depend (17) on the degree of involvement of the methine bridge bonds in the mode (18,19) consistent with the view that the core expansion is accommodated mainly at the methine bridges (7b). This core size effect is the basis of the well-known sensitivity of heme RR spectra to the spin-state of the Fe ion (20,21). Conversion from low- to high-spin hemes lengthens all the Fe-ligand bonds, including those to the pyrrole N atoms, thereby expanding the porphyrin core (2). The expansion is greatest when the high-spin Fe atom is constrained to be in the heme plane via two equivalent (weak field) axial ligands. With only one axial ligand, as in deoxyHb, the iron moves out of the plane, allowing the porphyrin core to relax somewhat. For five-coordinate high-spin Fe^{II}, however, there is a significant doming of the pyrrole rings, as they follow the Fe atom movement, at least in the case of the 2-methylimidazole adduct (22) which serves as a model for deoxyHb. This doming appears to lower some of the porphyrin skeletal frequencies (17), thereby diminishing the differences expected from the altered porphyrin core expansion between six- and five-coordinate Fe^{II} (17).

For low-spin Fe^{II}hemes, the effect of back donation from the filled d_π orbitals into the empty porphyrin π^* orbitals is clearly seen in substantial deviations of several of the skeletal frequencies from the values expected on the basis of the core size (17). The deviations are symmetry-specific, and are plausibly related to the nodal pattern of the porphyrin e_g^* orbitals (17). When the axial ligands are systematically varied to increased their π-acceptor ability, thereby reducing the extent of Fe^{II} backbonding to the porphyrin, these deviations diminish progressively (23). For especially strong π-acceptors, such as O_2 (20) and NO, (24) back donation to the porphyrin is fully surpressed, and the frequencies fall in the range expected for low-spin Fe^{III}, as noted early on (20,21) for oxyHb.

One RR band, near 1370 cm^{-1}, which is especially prominent upon Soret excitation, appears to be a marker for the Fe oxidation state (20,21). It is found close to 1375 cm^{-1} for Fe^{III} hemes, whether low- or high-spin, close to 1380 cm^{-1} for low-spin Fe^{IV} (25) and close to 1360 cm^{-1} for low- or high-spin Fe^{II}. For low-spin Fe^{II}, however, the frequency shifts up, toward the Fe^{III} value, when the axial ligands are π acceptors (23). This band is known from its ^{15}N shift (26) to be primarily the breathing mode of the pyrrole C-N bonds. It appears to be sensitive mainly to the polarizing power of the Fe ion, as controlled by its oxidation state, but also to the occupancy of the porphyrin e_g^* orbitals. The frequency shifts down upon porphyrin ring reduction to the mono- and di-anion (27). The near coincidence of the low- and high-spin Fe^{II} frequencies is probably coincidental, the higher polarizing power of low-spin Fe^{II} being compensated by its e_g^* back donation. It is interesting that the frequency is shifted to abnormally low values in cytochrome P_{450} (28,29) which is known to have cysteine thiolate as the fifth ligand. The thiolate is capable of donating its p_π electrons to the porphyrin π^* orbitals (30), thereby producing the frequency lowering.

Axial Ligand Modes

Although the most prominent RR bands of heme are porphyrin skeletal modes, careful searches of the lower-frequency region, have revealed a number of candidates for Fe-axial ligand stretching modes, which in several cases have been confirmed by isotopic substitution (6). For Hb and Mb, the most relevant axial ligands are imidazole and O_2. Fhe $Fe-O_2$ stretching band of HbO_2 was located at 567 cm^{-1} in an early study by Brunner (31). It is weakly enhanced via resonance with the porphyrin π-π^* transitions; (32,33) because of the competion between the O_2 and the porphyrin for the Fe d_π electrons; stretching of the $Fe-O_2$ bond can plausibly modulate the π-π^* energies (32). Likewise the Fe-NO stretch in NOHb (34,24) and the Fe-CO stretch in COHb (33,35) are enhanced via the π-π^* transitions. The Fe-NO stretch is responsive to the presence or absence of a trans axial ligand (24), and its frequency helped to establish (24) that the Fe-imidazole bond is broken, or greatly weakened, in the α chains of human NOHb when it is switched to the T quaternary state via the addition of inositol hexaphosphate (IHP) (36,37). But this does not appear to happen for carp NOHb, despite the switch in quaternary state (38). Surprisingly, the $Fe-O_2$ and Fe-CO frequencies are not

very sensitive to the O_2 or CO affinity of the heme. Thus no difference in Fe-O_2 frequency was seen for the O_2 adducts of Mb substituted with a range of modified porphyrins, having different O_2 affinities (39), and no significant frequency shift was observed upon switching the mutant O_2Hb's, Kansas and Milwaukee, between R and T quaternary states (40). Likewise COHb Kansas showed no alteration of the Fe-CO band upon IHP addition, although IHP addition to carp COHb did produce a shoulder on the low-frequency side of the band (33). Slight shifts in frequency, down for Fe-O_2 (41) but up for Fe-CO (33) have been seen in O_2 or CO complexes of "picket fence" porphyrin, when the axial base is a sterically hindered imidazole. It appears that the affinity differences commonly observed for O_2 and CO heme adducts are mostly due to factors other than the Fe-O_2 or Fe-CO bond strength *per se*.

The Fe-imidazole stretching frequency in five-coordinate Fe^{II} complexes has been identified with a band at ~220 cm^{-1}, via ligand perdeuteration in the 2-methylimidazole adduct (42), and ^{54}Fe substitution in this adduct (44) and in Mb (43). An alternate assignment, to Fe-N(pyrrole) stretching, has been advanced (45), and the reported 1.5 cm^{-1} ^{15}N shift (46) does indicate appreciable pyrrole involvement, but the specific ligand perdeuteration shift strongly supports the Fe-ImH (ImH = imidazole) assignment. This band is variable in frequency, in a manner indicating an important role for the ImH H-bonding (47). The frequency is ~205 cm^{-1} in non-aqueous solvent (47) or aqueous detergent (43), ~220 cm^{-1} in water, and ~240 cm^{-1} when the ImH proton is removed with strong base in non-aqueous solvent (47). Its high frequency, 242 cm^{-1}, in reduced horseradish peroxidase (48) is consistent with absorption spectral evidence (49) that the proximal imidazole is deprotonated, or strongly H-bonded.

In Hb and Mb the Fe-ImH band is at ~220 cm^{-1}, (42,44) consistent with moderate H-bonding to a peptide carbonyl O atom (50,51). However the band is asymmetric on the low-frequency side, and Nagai and Kitagawa (52) have shown, using valency hybrid Hb's, that the α and β chains have appreciably different Fe-ImH frequencies, 207 and 222 cm^{-1}, in the T state. These relax to 220 and 224 cm^{-1} in the R state. This is the first evidence for a specific structural effect at the heme group of the T quaternary state; it is expressed mainly in the α chains. Nagai and Kitagawa interpreted the frequency shifts in terms of mechanical tension on the Fe-ImH bond, exerted by the protein (3,4). However the shifts can also be interpreted (47) in terms of reduced ImH H-bonding in the T state, which could plausibly provide an electronic mechanism for the lowered O_2 affinity (53,54).

COHb and O_2Hb Photodynamics

The dynamics of CO or O_2 dissociation from Hb, which can be induced very rapidly by the absorption of light, can be expected to provide insight into the connection between heme ligation and protein structure change. It is known from the study of picosecond laser-induced absorption transients that a spectrum resembling that of deoxyHb (55) is generated within 10ps (55-57) of photolyzing COHb. The RR spectrum of this species observed with 10ns (58-60) or 30ps (61,62) laser pulses, that serve both to photolyze the sample and generate the RR spectrum, likewise resembles that of deoxyHb, but there are slight frequency downshifts (60-62) which are consistent with the Fe^{II} ion being high-spin but remaining in the heme plane. The <30ps conversion to a high-spin state implies that photolysis proceeds via intersystem crossing of the photoexcited COHb to a dissociative high-spin ligand-field state of the Fe^{II}; rates of thermal spin conversion of Fe^{II} complexes are in the 10-100 ns range (63).

Because the frequency differences between domed and planar high-spin Fe^{II} hemes are small (17), it is difficult to be quantitative about the degree of out-of-plane displacement of the Fe atom in the photointermediate (62), but the observed frequency shifts establish that the heme has not relaxed to its fully out-of-plane deoxyHb structure. This relaxation was determined (64) to have taken place within 300ns in a flow experiment, in which the interaction time of the COHb sample with the laser was determined to be <300ns; in the resulting photoproduct spectrum the frequencies were within 1 cm^{-1} of the deoxyHb frequencies. Since nuclear motions are very rapid, and there is no reason to expect an activation barrier for out-of-plane movement of high-spin Fe^{II}, the relatively slow relaxation observed for Hb is suggested (62) to reflect a protein conformation change to which the Fe movement is coupled. This Fe-coupled tertiary structure change may well be linked in turn to the subsequent R → T quaternary change, which is known to occur in tens of μs (65). X-ray crystallography (66) shows that Hb deligation is accompanied by an appreciable (~1Å) shift of the entire F helix, to which the proximal imidazole is attached. This large scale motion could well require tens of ns. Importantly, photolysis of COMb produces a prompt (10ns) photoproduct RR spectrum which does not differ significantly from that of deoxyMb (67). Thus only for Hb does the Fe out-of-plane displacement appear to be coupled to a slow protein conformation change, which is plausibly a link in the mechanism of cooperativity.

In addition to the core-size sensitive bands, the oxidation-state marker band also shows a downshift (1356 cm^{-1}) relative to deoxyHb (1358 cm^{-1}) in the prompt COHb (but not COMb (67)) photoproduct (60). Similar downshifts have been noted for deoxyHb's modified to stabilize the R quaternary state (68) and were interpreted as evidence for differential π donation into the porphyrin e_g^* orbital by a nearby phenylalanine residue. Perutz (3) has suggested instead that the downshift is associated with the strengthened R-state Fe-ImH bonding revealed by the Fe-ImH stretching frequencies (52). This interpretation is consistent with the <300ns COHb

photoproduct spectrum (64) which shows the downshifted 1356 cm^{-1} frequency, although the core-size frequencies have relaxed, while the Fe-ImH stretching band is symmetric and is located at 222 cm^{-1} as expected for the R state. However Lyons and Friedman (69) report pulse-probe measurements on photolyzed COHb showing that most of the 1356 cm^{-1} relaxation occurs with a time constant of ~800ns, *before* the T → R switch, while the remainder relaxes in a later (T → R) transition. The nature of this ~800ns tertiary transition remains to be determined.

The photoproduct of O_2Hb generated with relatively weak (5 nJ)~ ~50ps pulses from a synchronously pumped dye laser has been reported (70) to give the same RR spectrum as the COHb photoproduct (9). When generated with shorter (30ps) and stronger (200 nJ) pulses from a YAG laser, however, the O_2Hb photoproduct spectrum is quite different (71), showing substantial lowerings of the skeletal porphyrin frequencies. This spectrum is interpreted as arising from an electronically excited state of deoxyHb, with appreciable π-π^* (possibly triplet) character, consistent with the observation by Hochstrasser and coworkers (72), that photolysis of O_2Mb, but not COMb, gives rise ao a rapidly relaxing (<90ps) absorption transient, suggested to be a deoxyMb electronically excited state.

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