Tr. J. of Chemistry
22 (1998) , 103 – 108.

Heme Transfer Reactions: An Important Prerequisite for Synthetic Oxygen Carriers

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Received 29.07.1996

Electronic changes of iron- and metal free porphyrins are reviewed in light of their importance to modify the ferri/ferro reduction potential and energy dissipation between ligated carbon monoxide and the porphyrin chelate. Substitutions at positions 2 and 4 are effective ways to exercise this influence. Other measures include exposure to water and modifications of axial ligation, including the 'three-ligand' case. These measures will also influence the stability of recombined hemin-protein entities, as apparent from the kinetics of heme-transfer from a modified donor protein to apomyoglobin, but the stability appears to be related to the bulkiness of 2,4-substitutional groups rather than to electron availability. It is suggested that the altered stability is a secondary effect from enhanced exposure to water in the crevice.

Introduction

The prosthetic group and functional center of hemoproteins is called heme. Heme or iron-porphyrin is a metallo-organic chelate with the ability to facilitate electron transfer, catalyze redox reactions with O_2 and H_2O_2, interact with NO, and reversibly bind molecules, O_2 and CO, during biological transport. The electronic properties of heme and therefore the function are governed by the surrounding protein, by porphyrin ring substituents, and by exposure of the porphyrin to the solvent, water in biological tissues[1−3]. These properties are additive and spectroscopically we can trace the origin of observed changes, often

exercised as an altered binding of the sixth ligand to iron on the distal side. The 'three ligand' case is a special form where an extra amino acid comes within the normal Fe-N distance[3]. This effect is believed to be important for synthetic mutants of hemeproteins[4].

The present work reviews electronic changes and the related stability of native and recombined hemeproteins. It is of relevance to know if recombined proteins form active and stable entities.

Experimental

Leghemoglobin (LegHb) a, c_1, c_2, d_1 and d_2 were isolated from soybean nodules[5−7]. Horseradish peroxidase (HRP) A2 and C2 were prepared from the root[6−8]. Cytochrome C peroxidase (CCP) was a gift from Dr. Takashi Yonetani, University of Pennsylvania. Chloroperoxidase was purchased from Sigma. Finally, Earthworm hemoglobin (EarthwormHb) was purified from decapitated worms[9].

Porphyrin free proteins, including apo-Myoglobin (apoMb) were prepared from native holoproteins by heme removal[6,7]. All apo-proteins have a natural affinity for heme at normal pH and artificial hemeproteins were prepared by spontaneous recombination with synthetic forms of 2,4 substituted iron-porphyrins[6,7].

Heme transfer was followed by UV-VIS spectroscopy and analyzed by fitting appropriate rate-equations to observed intensities[7]. Infrared spectra of the CO compounds were obtained with a Mattson Cygnus spectrometer[10].

Results and Discussions

Ligated carbon monoxide is a sensitive monitor of electronic changes and Figure 1 shows how substitutions at the 2,4 positions modify the ferric/ferrous reduction potential and the ν_{CO} vibrational frequency for the carbonyl form of heme in different proteins and with different proximal ligands[6,11].

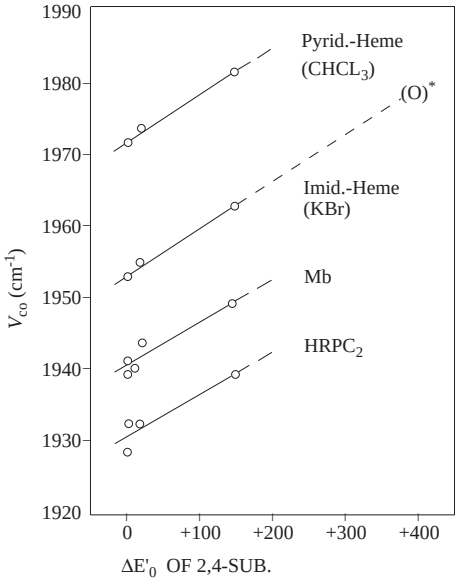


Figure 1. Vibrational frequencies for CO ligated to iron in heme as a function of changes of the ferric/ferrous reduction potential. Data are shown for different fifth ligands of free heme (pyridine and imidazole), different solvents ($CHCl_3$ and KBr) and two different proteins (myoglobin, Mb, and horzeradishperoxidase C_2, HRP-C_2).

Properties of crucial importance for catalytic reactions, such as energy dissipation during ligation and the fine-tuning of electron delocalization are analogous to metal adsorbent-adsorbate systems[10,12,13]. It appears that damped charge oscillations between distal CO and the porphyrin π-electron system can explain the observed relation between the position of the vibrational band and its half-band width[6,11]. A lower ferric/ferrous reduction potential induces a higher occupation of the first unoccupied orbital, CO $2\pi^*$, which reduces ν_{CO} and also opens the channel for charge transfer via iron orbitals. This is illustrated in Figure 2. Coupling to low frequency modes, excited by modest temperature changes, constitutes another channel for energy dissipation. This mechanism explains the observed intensity variations in ν_{CO} and Q_0, a vibronically coupled UV absorption band[10,12,13]. In Figures 3a-b we show a characteristic temperature dependence of around 200 cm^{-1} which is the measured value for the Fe-proximal histidine vibration, hence we can understand that thermal excitations on one side of iron will effect the bonding on the other side. The heterogeneous analogue to this is the effect low frequency adsorbent vibrations and phonons has on chemisorption.

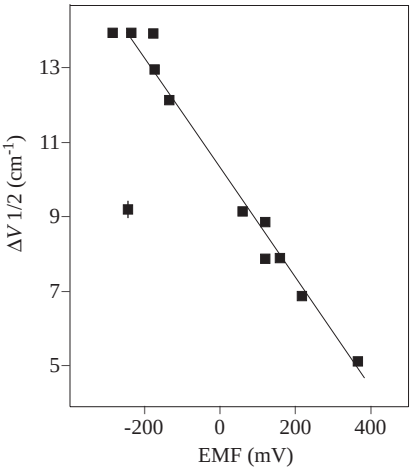
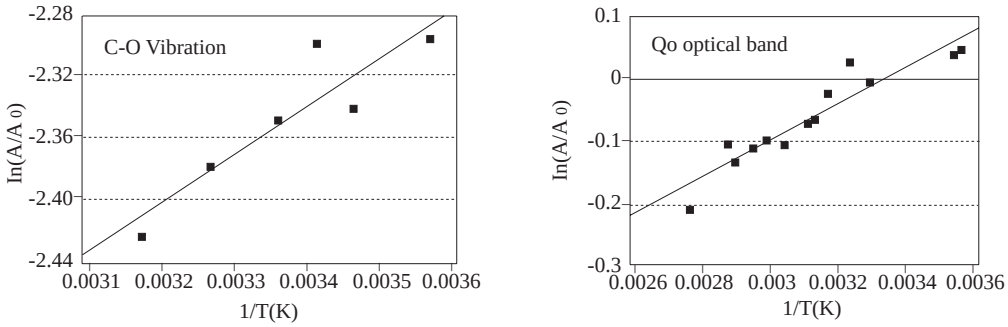


Figure 2. Half band width of the CO vibrational band for CO-heme in different proteins vs. the ferric/ferrous reduction potential.



(a) **(b)**

Figure 3. Temperature dependence of the intensity of (a) the C-O vibrational band of CO-LegHb and (b) the Q_0 optical band of Mb-CO, vibronically coupled to the CO ligand. The slopes correspond to (a) 201 cm^{-1} and (b) 226 cm^{-1}.

Hemoproteins have limited stability and are constantly being removed and replenished by the liver, spleen, and bone marrow. This denaturing process is a two step reaction, the first step is oxidation to Fe(III) or porphyrin oxidation and the second step is release of heme from the protein which may then irreversibly denature. One can model this process with transfer reactions for the prosthetic group between a donor holoprotein and an acceptor apoprotein[7,9,14]. Figure 4 shows how the dominant absorption band (Soret) of heme bound to a hemeprotein diminishes over time when a heme free acceptor protein is added. This means that there is an equilibrium between protein coordinated heme and free heme in solution and that free heme recombines with the new apoprotein if the energetics so favor.

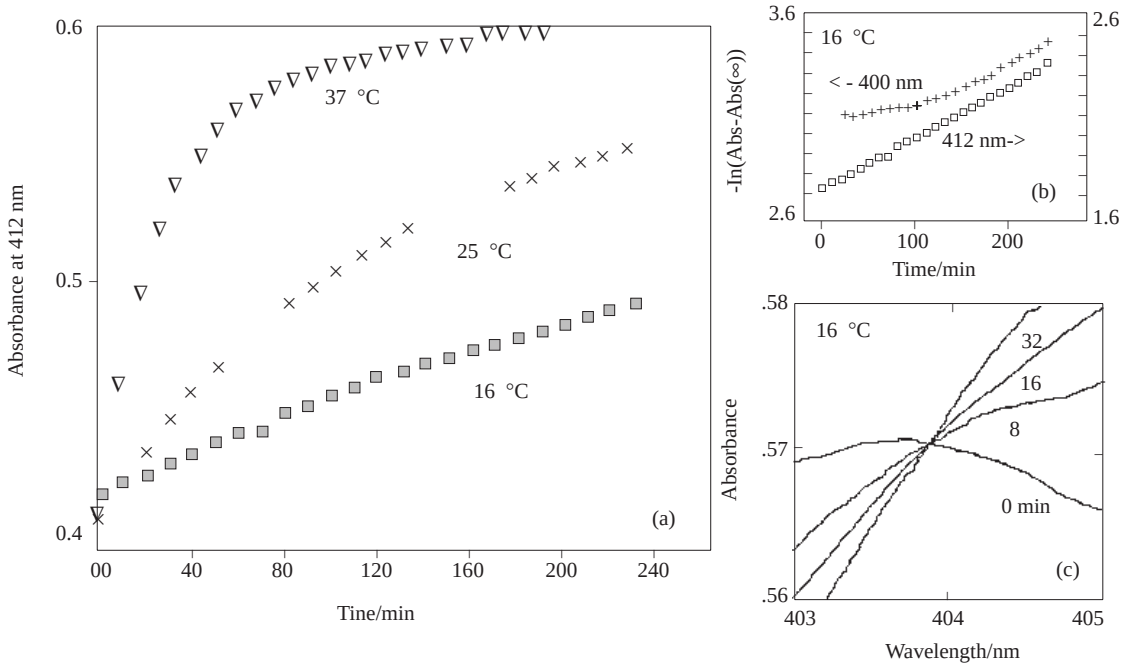


Figure 4. (A) Absorbance changes during heme release from leghemoglobin at three temperatures. Apomyoglobin was added as an acceptor at t=0 and we observe the effects of slightly different positions of the main absorption bands of heme in leghemoglobin and heme in myoglobin. (B) Semilogarithmic plot of courses at two wavelengths. (C) Isosbetic point during the initial phase.

A successful synthetic analogue for whole blood would be a welcomed addition to the clinic, given that the entangled prosthetic group is stable in the protein 'crevice' and that its properties can be tuned to mimic those of natice oxygen carrying hemoproteins, myoglobin (Mb) or hemoglobin (Hb). The problems of fine tuning the oxygen affinity, reduction of the oxidation products, and stabilizing the heme-protein interaction, are important and active research interests[15,16].

We have found that most hemoproteins exist in two states: (i) an active stable form with the iron anchored to the protein via its fifth ligand on the proximal side and (ii) an inactive less stable form with an altered position of the prosthetic group in the crevice with no anchoring bond. These configurations influence the kinetics of heme release as illustrated in Figure 5 and in Table 1. The crucial test was the observation of comparable separation rates for the inactive phase and a holoprotein with a metal-free porphyrin, the latter obviously lacking the iron-protein bond. The apparent small activation energy for heme uptake by apoproteins from solution should more accurately be replaced by a peak in the barrier for release when one layer of water molecules has formed between the porphyrin disc and the protein surface[17]. A peak in the

barrier at this position for release from either the donor or the acceptor protein creates an apparent minima for solvated heme.

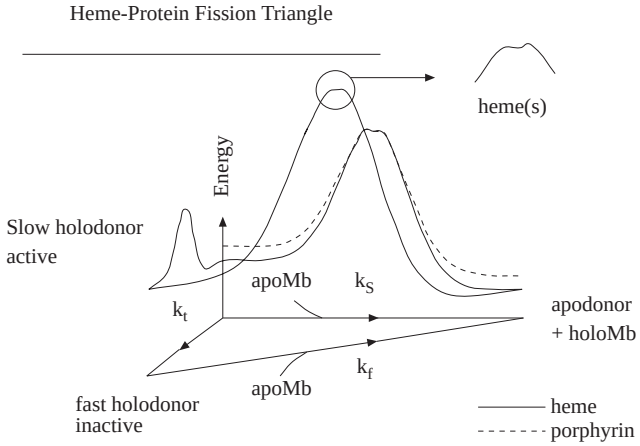


Figure 5. Energy diagram for heme transfer from two observed forms of a holoprotein donor to an acceptor protein, apo-Mb. The slow to fast holoprotein donor conversion, k_t, is partly irreversible, k_s and k_f are the rate constants for heme transfer from the slow (active) and fast (inactive) forms, respectively. The dashed line represents the barrier for the release of metal free porphyrins.

Table 1. Kinetic parameters for the slow phase release from native hemoproteins at 25° C.

Protein	Rate Constant 1/min.	Pre. Exp. Factor	$\Delta H°$ kJ/mol	$\Delta S°$ J/mol.K	$\Delta G°$ kJ/mol	Range °C
LegHb *a* (> 23° C)	0.00710	9.5E + 20	130	117	95	23-37
LegHb *a* (> 23° C)	0.00630	8.9E + 13	90	-20	96	7-23
HRP A2	0.00047	1.1E + 21	137	117	102	14-45
CCP	0.00017	1.5E + 73	437	1110	105	25-37
HRP C2	0.00024	5.2E + 21	131	91	104	30-45
Hb A	0.00773	7.0E + 20	120	82	95	25-42
Chloro-peroxidase	0.00190	n.a.	n.a.	n.a.	99	25
Earth-worm Hb	0.01690	n.a.	36	-195	94	25

Studies have shown that the effects of protein and peripheral substituents were additive on the electronic structure of heme but the stability of the chelate in the protein sets limits to artificially tuning the enzymatic function by modifications of porphyrin substituents. Bulky groups give less stable proteins, particularly if the crevice or 'pocket' is too narrow or too wide, and can result in increased exposure of heme to the polar solvent, reducing the electrochemical potential, and lowering the barrier for heme-protein fission.

We continue our work to unify the different rules by which nature tune the properties of hemoproteins to different functions[18], without overlooking the requirement that the prosthetic group and the protein must

unite and stay together to give an active enzyme. These rules should be useful for development of synthetic blood substituents, and for commercial catalytic processes with O_2, H_2O_2, NO, and CO.

Acknowledgement

We gratefully acknowledge our ongoing collaboration with Dr. Olle Edholm from the Physics Department at the Royal Institute of Technology in Stockholm.

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