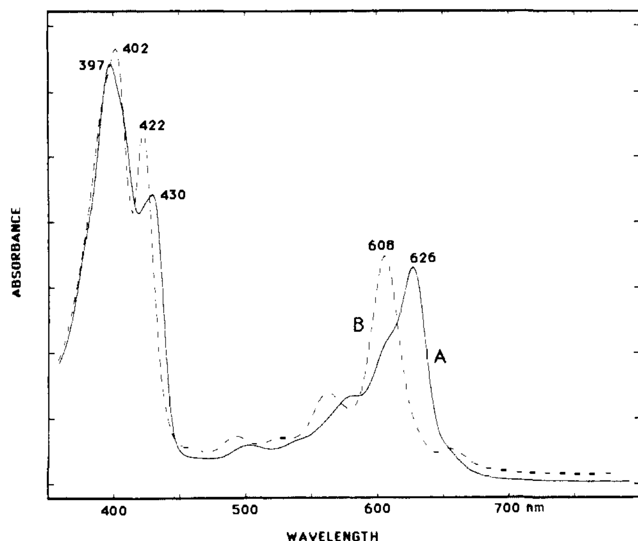

Figure 1. Electronic absorption spectra, in pyridine, of (A) predominantly ZnEdIBC (3) and (B) predominantly ZnVIBC (4).

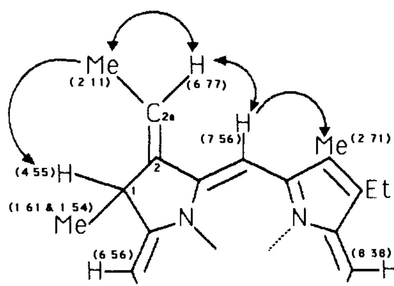

Figure 2. Proton NMR chemical shifts (in parentheses; solution in $CDCl_3$ + pyridine-d_5; Nicolet NT-360, 360 MHz) and network of NOE connectivities for ZnEdIBC (3). Coupling constants (Hz): $J_{H1-Me} = 7.3$; $J_{H1-H2a} = 1-2$; $J_{H2a-Me2a} = 7.1$ Hz.

When our compound **3** was treated with 2,3-dichloro-5,6-dicyanobenzoquinone, the optical and NMR spectra of the resulting oxidized product were identical with those of ZnPPheo (**1**), also confirming that the reduced product must still contain the 2-vinyl group.

The byproduct absorbing at 608 nm also oxidized to ZnPPheo (**1**), but at a slower rate. Logic suggested that the blue-shifted material should be zinc(II) vinylisobacteriochlorin (ZnVIBC, **4**), an isobacteriochlorin similar to that reported by Seely to be the major product. Catalytic hydrogenation of the ethylidene/vinyl mixture gave only one isobacteriochlorin (ZnEIBC, **2**), absorbing at 600 nm in CH_2Cl_2.

Careful spectrophotometric monitoring of the photoreduction showed that the 608-nm product was a precursor of the ethylidene compound **3**. Depending on the reaction conditions, the relative proportions of the two reduced products **3** and **4** was usually 50–90% in favor of the ethylidene compound. Milligram quantities of free bases **5** and **6** [obtained by treatment of the zinc(II) ethylidene/vinyl mixture with trifluoroacetic acid] were separated for NMR by reversed-phase HPLC. Formation of the ethylidene compound **3** from its vinyl precursor **4** is merely an allylic shift, probably a consequence of the basic reaction conditions. Also isolated from the reduction reaction was a small amount of zinc(II) methyl mesopyropheophorbide *a* (**7**), presumably produced by further rearrangement of the ethylidene material to chlorin.

Photoreduction to give predominantly ZnVIBC (**4**) was achieved using benzene as the solvent in place of pyridine and by cutting down the excess of DABCO. In this way, reduced products greater than 95% enriched in **4** were isolated on a large scale. The optical spectum of **4** obtained in this way is shown in Figure 1B; in benzene the long-wavelength absorption appears at 600 nm, while it is at 608 nm in pyridine.

Isolation of pure isobacteriochlorin also allowed an investigation of the stereochemistry of the photoreduction. The vinyl group was readily reduced with hydrogen and Pd/C to give ZnEIBC (**2**), which could be demetalated to EIBC (**8**). The best separations were accomplished with NiEIBC (**9**), obtained by nickel(II) insertion into EIBC (**8**). This nickel complex was separated by reversed-phase HPLC into three peaks, two of which (80% of product) were identical with the *cis*-NiEIBC (**9**),[5] and the other 20% we assign to the trans product. (One cis and one trans isomer have coincidental retention times.) The stereochemistry was confirmed by NMR spectroscopy. The two major resonances for each meso proton had chemical shifts identical with the corresponding meso protons of *cis*-NiEIBC (**9**), thus confirming the stereochemistry of the newly reduced ring as mainly cis. The stereochemistry of the isobacteriochlorin obtained by hydrogenation of the ethylidene/vinyl mixture was determined to be 70% trans and 30% cis in the same manner.

Apart from providing a simple synthetic route to ethylidene-containing macrocycles, the chemical ease with which the ethylidene compound **3** can be synthesized may have important biosynthetic implications. The ethylidene function is found in BChl *b* and *g* and in the chromophores from the phycobilins. Reduction of the carbon–carbon double bond in the pyrrole subunit followed by migration of the vinyl double bond to the ethylidene position is at least as likely as vinyl reduction to ethyl, followed by migration of the pyrrole carbon–carbon double bond to the ethylidene site; indeed, this last step would be thermodynamically unfavorable owing to loss of aromaticity in an equilibrium process.

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Pressure Dissociation of a Protein–Protein Electron-Transfer Complex

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There has been intense recent interest in the molecular mechanisms of electron transfer between physiological redox transfer partners and the mechanisms by which such proteins recognize and form chemically competent complexes with the observed high degree of specificity. We report herein the first determination of the partial specific volume change attending the formation of a protein–protein complex between the physiological redox transfer partners cytochrome b_5 and hepatic cytochrome P-450_{LM2}.

Cytochrome P-450 isozyme LM_2 and cytochrome b_5 solubilized from the hepatic endoplasmic reticulum have been shown to form a tight 1:1 complex in solution.[1,2,9] Formation of this complex is readily monitored by a large perturbation of the P-450_{LM2} optical spectrum that is attributed to a change in the spin state of the

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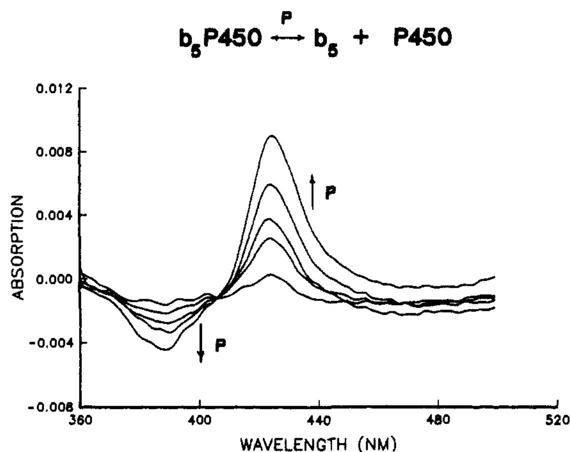

Figure 1. Pressure-induced optical difference spectra indicating dissociation of the cytochrome b_5–P-450_{LM2} complex. A reference spectrum obtained at atmospheric pressure was subtracted from spectra observed at elevated pressures. Representative traces at pressures of 200, 400, 600, 800, and 1000 bar are shown.

heme center from a low-spin ($S = {}^1/_2$) ferric iron to a high-spin $S = {}^5/_2$) state.[3]

Volume changes accompanying protein binding of substrates, ligands, or other macromolecules can be measured by the application of hydrostatic pressure.[4] In order to quantitate the volume change accompanying the formation of the physiological electron-transfer aggregate described above, a preformed complex of cytochrome b_5 and cytochrome P-450_{LM2} was subjected to increasing hydrostatic pressure in a steel optical cell with sapphire windows.[5] The purified protein preparations used for these experiments were electrophoretically homogeneous with purities of 32 nmol of cytochrome b_5/mg of protein and 16 nmol of cytochrome P-450_{LM2}/mg of protein. A spectrum was recorded from 350 to 500 nm at atmospheric pressure to serve as a reference. Figure 1 illustrates a family of difference spectra relative to this reference that are induced by a series of increasing pressures. Increasing dissociation of the cytochrome b_5–cytochrome P-450_{LM2} complex is represented by an increase in absorbance at 420 nm and a corresponding decrease at 390 nm as previously described.[3] A clean isosbestic point was observed at 406 nm, indicating that the observed pressure effects can be described by a simple two-state system. These optical changes were completely reversible as the pressure was lowered, in that spectra were exactly superimposable at equivalent pressures and an identical base-line spectrum was regenerated with no evidence for hysteresis. Protein viability was verified at the end of each experiment, with no evidence for denaturation of either protein nor the production of P-420, a commonly observed inactive form of cytochrome P-450_{LM2}. These pressure-induced optical changes were identical with those observed in a titration of cytochrome P-450_{LM2} with cytochrome b_5 conducted at atmospheric pressure. No spectral change was observed on pressurization of cytochrome P-450_{LM2}. However, pressurization of cytochrome b_5 alone showed a significant absorbance change in a difference spectrum with a maximum at 416 nm. This difference spectrum lacked the 390-nm trough, 420-nm peak, and 406-nm isosbestic point observed for the pressure-induced dissociation of the cytochrome b_5–P-450_{LM2} complex and represents pressure-induced solvation of the heme prosthetic group from cytochrome b_5. These pressure-induced spectral changes for cytochrome b_5 were subtracted from the difference spectra obtained with P-450_{LM2} in the presence of cytochrome b_5 with typical corrected spectra shown in Figure 1. Similar pressure experiments

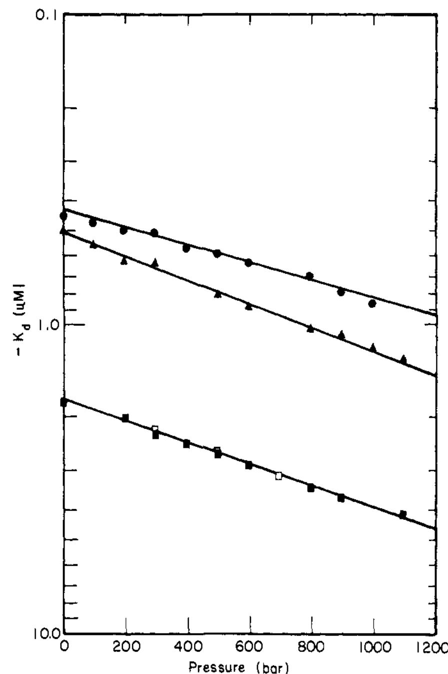

Figure 2. Pressure dependence of the dissociation constants for the cytochrome b_5–P-450_{LM2} complex yield volume changes for the macromolecular association. The protein concentrations used in all pressure experiments were 1.5 or 2.9 μM cytochrome P-450_{LM2} and 2.0 μM cytochrome b_5. Buffer conditions and binding volumes are (■) 50 mM KPO_4, pH 7.4 with open squares at 300, 500, and 700 bar denoting data obtained on pressure release, 25 °C, −19.4 mL/mol; (▲) 50 mM Tris-HCl, pH 7.4, 25 °C, −23.0 mL/mol; (●) 50 mM Tris-HCl, pH 7.4, 10 °C, −16.0 mL/mol. Tris-HCl has a negligable pressure dependence of pK whereas KPO_4 ionization is accompanied by a relatively large volume change due to electrostriction of solvent.[11]

were conducted at altered temperature and buffer conditions.

Any macroscopic equilibrium constant describing a two-state system can be written in terms of thermodynamic state functions as

$$-RT \ln K_{eq} = \Delta G = \Delta H - T\Delta S = \Delta E + P\Delta V - T\Delta S$$

where ΔG is the Gibbs free energy, ΔE the internal energy, P the pressure, ΔV the volume, T the absolute temperature, ΔS the entropy, and ΔH the enthalpy. Hence, the partial specific volume accompanying this binding equilibrium can be obtained as the slope of a plot of the logarithm of the equilibrium constant as a function of P/RT. The optical changes occurring due to the dissociation of the cytochrome b_5–P-450_{LM2} complex as shown in Figure 1 were used to calculate the macroscopic dissociation constants of this complex at each pressure. Figure 2 illustrates that a rigorously linear relationship was observed for all data sets, with an observed volume change for the dissociation of cytochrome b_5 and cytochrome P-450_{LM2} in Tris-HCl buffer (50 mM, pH 7.4, 25 °C) of −23.0 ± 1.3 mL/mol. This large value suggests the involvement of charge interactions in the formation of the complex. A slightly smaller volume change was measured at 10 °C, Figure 2.

In contrast to previous results,[9] a soluble form of rat cytochrome b_5 which lacks a hydrophobic tail domain[13] produced by expression of a synthetic gene in *E. coli* was found to bind cytochrome P-450_{LM2} with a dissociation constant of 3.5 μM in 50 mM Tris-HCl pH 7.4 at 25 °C. This soluble form of cytochrome b_5

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exhibited the same binding volume to P-450_{LM2} as the complete b_5 macromolecule.[14]

The formation of stable protein–protein complexes of the correct structural conformation is an important component of the observed specificity of electron-transfer events in physiological systems. Various authors have suggested the involvement of charge-pair or dipolar interactions in determining the final specificity and orientation of the constituent proteins in a productive aggregate.[6-8] In this model, the close juxtaposition of opposite charges contributes to the orientation energy necessary for correct positioning of the proteins and can aid in supplying the energy necessary for equal potential electron transfer between reactant- and product-activated complexes.[10] Separation of the aggregate would then result in the exposure of this surface contact domain to solvent. The effect of pressure on an ionization equilibrium can be qualitatively understood by considering this solvation process. The electrostriction of water around a bare charge results in a smaller system volume, and hence high pressure will favor the state with the greatest net charge exposed to solvent. Hence, the ionization of acetic acid, which represents a change in total solvated charge of 2 (COOH being uncharged and $COO^- + H^+$ representing two charges), is accompanied by a large decrease in volume (−11 mL/mol), whereas the ionization of a primary amine or an imidazole nitrogen (which results in no net change in system charge) is less affected by pressure (−1 mL/mol).[11,12] The understanding of relatively simple chemical ionizations has been extended to encompass the more difficult case of macromolecular dissociation by Weber and co-workers.[4] In the work presented in this paper, we extend measurements of multimeric protein dissociation to the case of a heterologous dimer of physiological redox transfer proteins. If complementary charge pairing occurs between cytochrome b_5 and cytochrome P-450_{LM2} at the protein–protein interface, one might expect a large volume change for the system on dissociation of the complex due to the solvation of exposed charges. To the extent that the separation of charge in the case of heterologous dimer dissociation is analogous to the ionization of acetic acid,[11] and assuming that solvent is completely excluded in the dimer interface,[7,8] our observed volume change for the cytochrome b_5–P-450_{LM2} couple of −23.0 mL/mol is consistent with the involvement of roughly two ion pairs in the association of these two proteins.[15] Such a conclusion is consistent with that reached from protein chemical modification studies[2] and illustrates a potentially powerful application of high-pressure spectroscopy for study of electron donor–acceptor complexes.

Acknowledgment. We thank Professor Gregorio Weber for many useful discussions, including insights into the interpretation of pressure-induced protein dissociations, Mary Beth McCarthy for assistance in protein purification, Dr. Ralph Murray for aid in computer programming, and Kris Ludington for construction of the high-pressure spectrophotometer system. This work is supported by NIH Grants GM31756 (SGS) and ES03600 (REW).

(14) Work is in progress which will characterize the interactions with a number of heme proteins with various mutant forms of soluble rat cytochrome b_5 that have been constructed by site-specific mutagenesis techniques.

(15) Typical volume changes previously observed for spin-state transitions in heme proteins are on the order of 5 to 15 mL/mol.[16-18] However, the pressure-induced dissociation of camphor and camphor analogues from cytochrome P-450_{cam} exhibits very similar volume changes regardless of the original ferric spin state distribution.[19] The dissociation volumes for substrates bound to cytochrome P-450_{cam} differ by only 2–7 mL/mol. This suggests that the conformational change which occurs during a spin-state transition in cytochrome P-450_{cam} is small. Small-angle X-ray scattering measurements have shown that the radius of gyration for the predominantly low-spin camphor-free form and predominantly high-spin camphor-bound form are nearly equal, 23.9 ± 0.2 and 23.7 ± 0.2 Å, respectively.[20] Numerous investigators have suggested that the ferric high-spin form of cytochrome P-450 exists as a five-coordinate species.[21-23] The low-spin form of the porphyrin-chelated ferric iron is six-coordinate with H_2O or OH^- providing the sixth axial ligand.[22-26] Since the active site of P-450_{LM2} is accessible to solvent[27] thus resembling cytochrome P-450_{cam}, it is reasonable to suggest that the conformational changes which occur during the ferric spin transition of P-450_{LM2} are also small compared to the cytochrome b_5–P-450_{LM2} dissociation.

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Electrochemistry of Heptylviologen in the Presence of Phosphatidylcholine Liposomes

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This paper describes novel preliminary results on the electrochemistry of 1,1′-diheptyl-4,4′-bipyridinium ion (heptylviologen, HV^{2+}) in the presence of 1-phosphatidylcholine (PDC) liposomes. Vesicles and liposomes are currently attracting considerable interest due to the structural similarity of their bilayer walls to biological membranes.[1] To the best of this author's knowledge this is the first report on electron transfer to or from a dimensionally stable electrode and vesicle-solubilized redox substrates.

The electrochemical behavior of heptylviologen dibromide in 50 mM phosphate buffer (pH 7) is not controlled by diffusion due to the water-insoluble nature of its two reduced forms ($HV^{\bullet+}$ and HV). Cyclic voltammetry of this solution yields two close reduction peaks and three oxidation spikes upon scan reversal. The reductive behavior is explained by two consecutive one-electron reduction steps to yield the cation radical and the neutral compound.[2] The sharp peaks on the oxidative scan result from the precipitation of the $HV^{\bullet+}$ and HV forms onto the electrode surface.[3]

The addition of a cationic surfactant, such as cetyltrimethylammonium chloride (CTAC), changes this electrochemical behavior (see Figure 1A). The cation radical, $HV^{\bullet+}$, is solubilized by the cationic micelles probably via coaggregation. Conversely, the solubilizing capacity of the CTAC micelles is overwhelmed by the fully reduced HV as can be concluded from the sharp spike on the oxidative scan. In the presence of PDC liposomes[4] (see

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