Observing the ^{1}H NMR signal of the myoglobin Val-E11 in myocardium: An index of cellular oxygenation

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ABSTRACT The ^{1}H NMR signal from oxymyoglobin, a low-concentration diamagnetic protein, is visible in myocardial tissue. The methyl group of the Val-E11 resonates in a clear spectral region at -2.76 ppm and responds to dynamic changes in cellular oxygenation. With CO, the signal shifts to -2.4 ppm. The Val-E11 peak assignment and its response to oxygen and CO agree perfectly with previous myoglobin solution studies. Intracellular oxygen level can now be determined *in vivo* with the signal intensity ratio of oxymyoglobin/deoxymyoglobin, reflected by the Val-E11 and His-F8 peaks in the ^{1}H NMR spectra. Moreover, protein structure–function relationship *in vivo* can now be probed.

Cellular oxygenation is a fundamental physiological parameter. Yet current techniques to detect it *in vivo* without invasive manipulation are severely limited by their sampling specificity and tissue localization. Current techniques measure the arterial–venous differences in hemoglobin saturation or substrate linked with NADH-dependent enzymes, such as lactate dehydrogenase (1). Electrochemical and optical techniques rely on catheterization, electrodes, or surface measurements (2, 3). Intracellular oxygenation in a local tissue region is indeed quite difficult to ascertain.

Although NMR methods have demonstrated the capacity to localize noninvasively important metabolite signals *in vivo* (4, 5), such as [^{31}P]phosphocreatine and ATP as well as the ^{1}H lactate signals, they have provided only indirect indicators of cellular oxygen levels. This laboratory has shown from perfused heart studies that the ^{1}H NMR signal of the proximal histidine NH in deoxymyoglobin (deoxyMb) is visible and is sensitive to changes in cellular oxygenation (6). Its visibility and response are predicated on the heme iron, which in the paramagnetic deoxygenated state, hyperfine shifts the resonance to a clear spectral region 75 ppm downfield of the water line (7). Upon oxygenation, Mb becomes diamagnetic and the signal disappears.

To obtain cellular oxygenation levels with only the deoxyMb signal poses a quantitation difficulty. Neither the total Mb nor the oxymyoglobin (oxyMb) concentration is known. A deoxyMb/oxyMb ratio would circumvent the obstacle and lead directly to a value of oxygen partial pressure in the cell. However, detecting the oxyMb, a diamagnetic protein, signal *in vivo* is a daunting proposition and appears to be untenable, especially in light of its low concentration and the interfering endogenous signals in the diamagnetic spectral region. To our knowledge, no NMR signal from a diamagnetic protein in intact tissue under physiological conditions has been reported.

We have, however, focused on the ring current shifted signal from the Val-E11 methyl group in oxyMb and report that under optimal pulsing conditions, it is indeed observable and responsive to incremental changes in cellular oxygenation. The signal gives us then a key parameter in measuring

cellular O_2 with *in vivo* NMR and indicates that a cellular protein, even in its diamagnetic state, is detectable. It is clear that the Val-E11 signal can now serve as both a functional and structural probe of oxyMb *in vivo*.

MATERIALS AND METHODS

Perfused Heart Preparation. Male Sprague–Dawley rats (350–400 g) were anesthetized by an intraperitoneal injection of sodium pentobarbital (65 mg/kg) and heparinized (1000 units) by injection into the femoral vein. The heart was isolated and perfused using a modified Langendorff technique (6). Hearts were perfused at 23–25°C at a constant perfusion rate of 11 ml/min, which was maintained by a peristaltic pump (Rabbit, Rainin, Woburn, MA). Heart rate and perfusion pressure were continuously monitored by means of a perfusate-filled cannula connecting the aortic cannula with a Statham P23XL strain gauge transducer and a Gould RS 3200 oscillographic recorder. Perfusion pressure under control conditions was 65–75 mmHg; hearts were beating spontaneously at a rate of 60–80 min^{-1}. Perfusate flowing from the pulmonary artery bathed the heart. An overflow tubing above the heart withdrew the perfusate for recirculation by the peristaltic pump. The total recirculation volume was 200 ml. The perfusion medium was a modified phosphate-free Krebs–Henseleit buffer containing 118 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, and 10 mM glucose. The perfusion medium was gassed with 95% O_2/5% CO_2. Ischemia was induced by stepwise reduction of the perfusion flow rate. Hypoxia was induced by equilibrating the perfusion medium with various ratios of 95% O_2/5% CO_2 and 95% N_2/5% CO_2, which were maintained by a gas-mixing pump (Wösthoff, Bochum, F.R.G.).

NMR. ^{1}H NMR spectra were collected on a 7-T 15-cm-bore GE Omega system equipped with a home-built ^{1}H/^{31}P probe. The ^{1}H/^{31}P observe coil was 25 mm in diameter. The ^{1}H 90° pulse was 36 μs, calibrated with 0.1 M NaCl. The deoxyMb (His-F8) spectra were acquired using 90° pulses and a 40-ms repetition time. For the oxyMb (Val-E11) signal, the acquisition time was set to 40 ms and the Ernst angle was specified as 18°. The parameters were based on the expected relaxation time (T_1) of the oxyMb Val-E11 γ-methyl signal in the cell, extrapolated from the solution carbon monoxymyoglobin T_1 and the estimate of the cellular Mb rotational correlation time (ref. 8 and unpublished data). A modified 1331-pulse sequence (9) was then used to suppress the water signal. A typical 10-min spectrum required 15,000 transients. The ^{31}P spectra were collected in alternate blocks. Each block required 256 scans and 6 min of signal accumulation. A 70° pulse angle and a 1.3-s repetition time were used. The ^{31}P 90° pulse was 57 μs. After apodization at the natural linewidth, the ^{1}H signals were referenced to the water line, 4.76 ppm at 25°C, which was calibrated against 2,2-dimethyl-2-silapentane-5-sulfonate. The ^{31}P signals were referenced to phosphocreatine as -2.35 ppm.

Abbreviation: Mb, myoglobin.
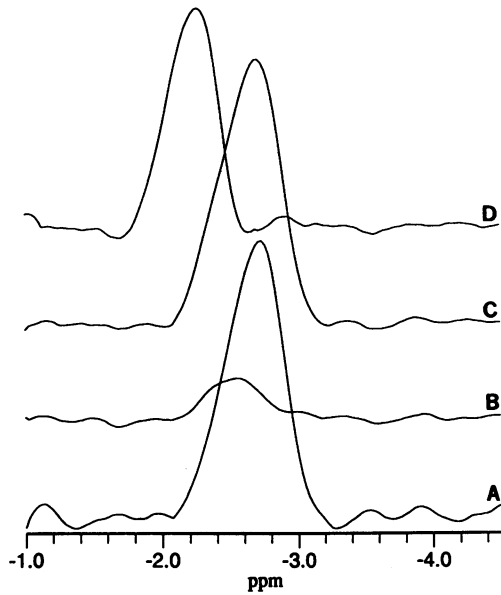*To whom reprint requests should be addressed.

FIG. 1. ¹H NMR spectra from perfused rat heart. Traces: a, signal appearing at −2.76 ppm is assigned to the oxyMb Val-E11 γ-methyl signal; b, upon deoxygenation, the signal disappears; c, on reoxygenation, the Val-E11 signal reappears; d, when CO is added to the system, the peak shifts to −2.4 ppm.

RESULTS AND DISCUSSION

¹H NMR spectra from perfused heart experiments are shown in Fig. 1. Even though the myocardial Mb concentration is about 0.1 mM, the γ-CH₃ Val-E11 signal is clearly detectable at −2.76 ppm with 10 min of signal averaging (Fig. 1, trace a). The chemical shift corresponds exactly to the one reported

previously (10). The sensitivity is clearly sufficient to follow the changes in cellular oxygenation. Under a totally ischemic condition (flow rate, 0 ml/min) the signal disappears (Fig. 1, trace b). Upon reoxygenation, the signal reappears (Fig. 1, trace c) at precisely −2.76 ppm. When CO is then added to the perfusion medium, the resonance shifts downfield to −2.4 ppm (Fig. 1, trace d). The spectral characteristics correspond perfectly with those reported for Mb solution studies (10).

The signal responds directly to varying oxygenation conditions in the perfused heart (Fig. 2 *Lower*). Ischemia was induced by stepwise reduction of flow: 11, 3, 2, 1, and 0 ml/min. In each step of the graded ischemia, the decrease in oxygen availability parallels a decrease in the Val-E11 signal intensity. In contrast, the proximal histidyl NH signal of deoxyMb (6) varies inversely (Fig. 2 *Upper*). The dynamic interaction between the Val-E11 and His-F8 signals with varying oxygen tension is demonstrated in Fig. 3. Based on a 100% signal at the totally ischemic and fully oxygenated conditions, respectively, Fig. 3 indicates that these signals reflect the dynamic changes in the oxygenation. No extracellular Mb contributes to the NMR signal (6).

The Val-E11 and the His-F8 signals lead directly to the intracellular partial pressure of oxygen, calculated from the half-saturation pressure (P_{50}) of Mb: $Po_2 = (oxyMb/deoxyMb) \times P_{50}$ (11) (Fig. 3). Since the Mb is fully saturated in the initial condition and the Mb molecule is completely NMR visible (12), the Val-E11 signal itself can reflect the cellular oxygenation. As the intracellular oxygen tension drops, the phosphocreatine/ATP ratio (13) remains constant until the P_{50} of Mb is reached (Fig. 4). Utilizing only the His-F8 signal yields similar results (12). Our data then indicate that Mb and high-energy phosphorous signals respond asynchronously to intracellular oxygen changes, con-
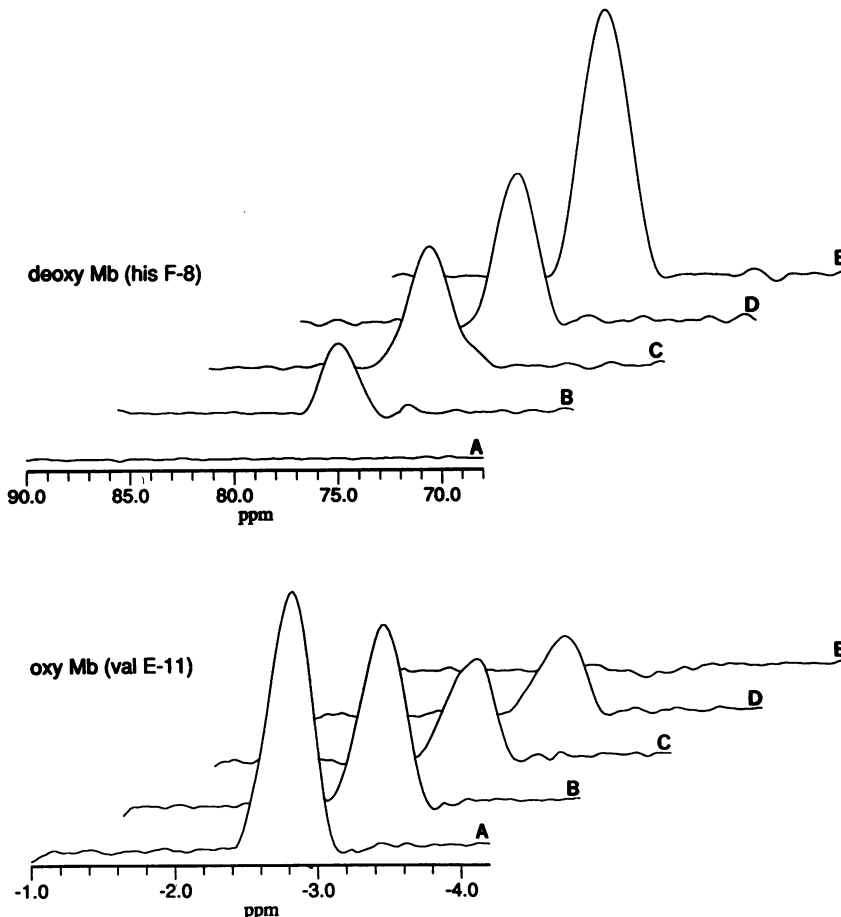

FIG. 2. ¹H NMR spectra of oxyMb and deoxyMb in isolated perfused rat heart under various ischemic conditions. Hearts were initially perfused at a constant perfusion rate of 11 ml/min (traces A), the perfusion rate was then decreased stepwise to 3 ml/min (traces B), 2 ml/min (traces C), and 1 ml/min (traces D), and subsequently the perfusate flow was stopped completely (traces E). After an equilibration time of 5 min, two ¹H spectra were taken at each step. Pulsing conditions for the first spectrum were optimized for the Val-E11 γ-methyl protons (*Lower*); pulsing conditions for the second spectrum were optimized for the His-F8 Nδ proton (*Upper*). The signals respond inversely to the varying oxygenation conditions; Val-E11 marks the oxygenated His-F8 the deoxygenated state.
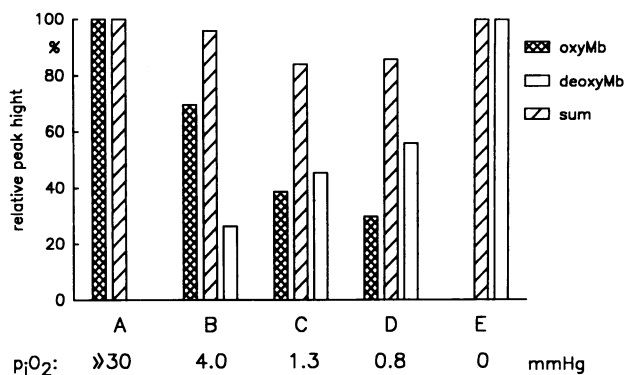
FIG. 3. Changing intensities of the Val-E11 and His-F8 signals under various ischemic flow rates, as described in Fig. 2. The 100% oxygenated and deoxygenated conditions were assumed for initial 11 ml/min (bars A) and the final 0 ml/min (bars E) flow rates, respectively. The graph clearly shows the dynamic equilibrium between these two peaks. In the graph, the sum bar reflects the total normalized ^{1}H NMR signal intensity from both the oxyMb and deoxyMb. Data in bars C and D indicate that the sum is only 85%, instead of the expected 100%. However, the current accuracy of measuring these peaks and the potential incomplete O_2 equilibration over the total acquisition period may account for the slight discrepancy. The corresponding intracellular Po_2 (pio_2), calculated from the ratio of oxyMb/deoxyMb and a P_{50} of 1.5 mmHg (3), is shown also. The intracellular Po_2 value given for bars A is based on >95% oxygen saturation of Mb; the value for bars E is taken to be 0.

sistent with the contrasting P_{50} values of Mb and cytochrome oxidase (14, 15). Clearly, the intracellular oxygen level can now be measured in vivo with the ^{1}H NMR signals of oxyMb/deoxyMb and key physiological questions centering on the role of Mb in facilitating oxygen diffusion in the cell
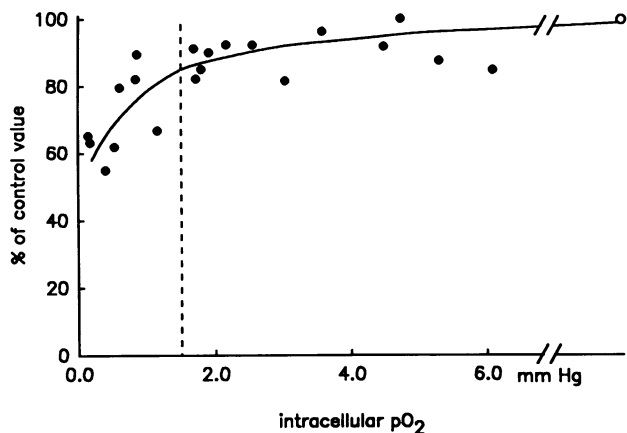

FIG. 4. Phosphocreatine/ATP ratio versus intracellular Po_2, which was determined from the ^{1}H NMR Mb signals of Val-E11. Under the initial condition, Mb is fully saturated with oxygen. It is also completely visible. Hypoxia was induced by stepwise reduction of the O_2 in the gas mixture. At each step, a ^{31}P spectrum was collected. The phosphocreatine/ATP ratio was obtained from the ^{31}P spectra and was normalized to 100% under a reference well-oxygenated condition (o). As the intracellular oxygen tension decreases, the phosphocreatine/ATP ratio remains relatively constant until the Mb P_{50} (1.5 mmHg) is reached (●).

(15) and the regulation of oxygen metabolism under normal and pathophysiological conditions (16, 17) can begin to be addressed.

Our results also have a direct impact on understanding the structure and function of proteins in their cellular environment. The relationship between structure and its function is a key issue central to many protein solution studies. Whether such solution studies have any direct bearing on proteins in their cellular environment remains an open question. Our data, however, suggest that contrasting structures may exist in vivo and in vitro. Although the ^{1}H NMR deoxyMb solution spectra exhibit broad resonances in the vicinity of −2.76 ppm, no signal has ever been detected in vivo in this same spectral region upon deoxygenation, even after prolonged signal accumulation. These resonances may be broadened severely in vivo (unpublished data) or reflect the contrasting protein structure in solution versus in the cell. Further work is still required to assign these resonances, which have at most a two-proton intensity, before definitive conclusions can be drawn.

Nevertheless, our study demonstrates that ^{1}H NMR can discern in vivo the main function of Mb, binding oxygen, and can now potentially probe the associated protein structure. The work then opens a way to probe protein function under physiological conditions and to examine the structure-function relationship in vivo.

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