Expression, Purification, and Properties of Recombinant Barley (Hordeum sp.) Hemoglobin

OPTICAL SPECTRA AND REACTIONS WITH GASEOUS LIGANDS*

(Received for publication, December 23, 1996, and in revised form, May 2, 1997)

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A cDNA encoding barley hemoglobin (Hb) has been cloned into pUC 19 and expressed in Escherichia coli. The resulting fusion protein has five extra amino acids at the N terminus compared with the native protein, resulting in a protein of 168 amino acids (18.5 kDa). The recombinant Hb is expressed constitutively. Extracts made from the bacteria containing the recombinant fusion construct contain a protein with a subunit molecular mass of approximately 18.5 kDa comprising approximately 5% total soluble protein. Recombinant Hb was purified to homogeneity according to SDS-polyacrylamide gel electrophoresis by sequential polyethylene glycol precipitation and fast protein liquid chromatography. Its native molecular mass as assessed by fast protein liquid chromatography-size exclusion was 40 kDa suggesting that it is a dimer. Ligand binding experiments demonstrate that 1) barley Hb has a very slow oxygen dissociation rate constant (0.0272 s⁻¹) relative to other Hbs, and 2) the heme of ferrous and ferric forms of the barley Hb is low spin six-coordinate. The subunit structure, optical spectrum, and oxygen dissociation rate of native barley hemoglobin are indistinguishable from those obtained for the recombinant protein. The implications of these kinetic data on the in vivo function of barley Hb are discussed.

The recently discovered nonsymbiotic plant hemoglobins constitute a new class of protoheme proteins that are expressed at low concentrations, on the order of 1–20 μmol per kg wet weight tissue, in non-nodule, often rapidly growing or rapidly metabolizing tissues such as roots, stems, or germinating seeds of monocotyledonous (1) and dicotyledonous plants (2, 3).

Nonsymbiotic plant hemoglobins are functionally and genetically distinct from the familiar symbiotic plant hemoglobins. Symbiotic hemoglobins are expressed in the cytoplasm of bacteriocytes of nodules formed in symbiotic association between legumes and the bacterium Rhizobium (3, 4) or between the actinomycoete Frankia and variety of non-leguminous woody dicotyledonous plants. In a unique instance, the bacterium Rhizobium nodulates a non-legate Parasponia andersonii (3). Although symbiotic hemoglobins show large differences in their gaseous reactions, all have in common rapid, almost diffusion-limited combination with oxygen and moderate rates of oxygen dissociation, so that they achieve very great oxygen affinity (5). Typically, nodule hemoglobins occur at up to millimolar concentration in the cytoplasm of the nodule cell, concentrations perhaps 10⁵ times greater than that of free oxygen. These properties, taken together, are ideally suited to facilitate a large flow of oxygen to the symbiosome at free oxygen concentrations as low as 10 μM, the oxygen pressure obtaining in the nodule cell.

Nonsymbiotic plant hemoglobin genes of both monocots and dicots fall into a single coherent gene family, distinct from the family of genes that encode symbiotic hemoglobins (6). In the unique instance of the nodulating tree, P. andersonii, a gene in the nonsymbiotic hemoglobin family encodes a hemoglobin whose pattern of expression and functional properties are those of a symbiotic hemoglobin (5, 6). Since nonsymbiotic Hb has now been demonstrated to occur in two of the major divisions of the plant kingdom, it is likely that a Hb gene is present in the genome of all higher plants (7–10), and it is suggested that most symbiotic Hbs genes arise by duplication of preexisting nonsymbiotic Hb genes (6).

Barley (Hordeum sp., a monocotyledonous cereal) nonsymbiotic hemoglobin is expressed in seed and root tissues under anaerobic conditions (1). Since the level of hemoglobin in barley root or aleurone tissue is of the order of 20 μM, we have developed an expression system in Escherichia coli to produce a barley hemoglobin fusion protein that closely resembles the native protein but differs in having five extra amino acids at the N terminus. This report addresses the expression, purification, and characterization of the fusion protein, in particular its reactions with oxygen and carbon monoxide and the configuration of the heme pocket of the ferrous (deoxy) protein. Some properties of native barley hemoglobin are also described. Comparisons with other hemoglobins show that some of the binding properties of recombinant and native barley Hb with gaseous ligands are unique. Two, not necessarily mutually exclusive, functions have been proposed for nonsymbiotic hemoglobins, as sensors of oxygen concentration and as carriers in oxygen transport (6). The results presented here suggest a third possibility, namely electron transfer, possibly to a bound oxygen molecule. To our knowledge this report describes the first characterization of a nonsymbiotic monocot plant Hb.

EXPERIMENTAL PROCEDURES

Materials—pUC 19 plasmid (Canadian Life Technologies), into which barley root Hb cDNA (1) was cloned, was used as the source of the barley Hb cDNA. Linker-adapters were constructed by the University of Manitoba DNA laboratory. E. coli strain DH5-α (Canadian Life Technologies) was used as the host for both recombinant and nonrecombinant plasmids. Subcloning efficiency DH5-α competent cells (Canadian Life Technologies) were used for transformation and growth of plasmids. Enzymes for DNA manipulation and agarose were from New England Biolabs. All other biochemicals were from Sigma or Canadian Life Technologies.

Construction of Expression Construct—pUC 19 plasmid, into which Hb cDNA had been subcloned from Bluescript I (between the restriction sites SstI and XbaI, was used as the starting material. The proce-
dure resulted in the removal of the 5'-untranslated region from the cDNA to minimize the extra amino acids of the final recombinant protein. The pUC19 ATG was used as the starting codon. The insert was removed from the plasmid and then reinserted into pUC19 in such a way as to remove the 5'-region and have the coding sequence in the correct orientation.

The plasmid containing the insert (approximately 100 µg) was digested for 6 h with SstII (which cuts the insert at 7th and 10th base pair inside the coding sequence) (7) and then dephosphorylated with calf intestinal alkaline phosphatase. The 8-mer (single-stranded adapter, ATCCGCAGG) was then phosphorylated and allowed to anneal with the 14-mer (single-stranded adapter, AGCTTACGGCCG) to form a link-er-adaptor containing an SstII site at one end, HindIII site at the other end, and a NotI site in the middle. The adapter was then ligated to the insert end of the SstII-digested plasmid. The plasmid was then digested with SstI releasing the insert cDNA (with the linker-adapter ligated to it forming a HindIII site at the end). The insert was separated from the plasmid by agarose gel electrophoresis and purified using GeneClean II Kit.

Nonrecombinant pUC19 (approximately 100 µg) was double-digested with SstI and HindIII and then dephosphorylated. The insert was then ligated with the nonrecombinant pUC19, and the resulting ligation mix was used to transform DH5-α E. coli cells.

Transformation and Screening of Recombinant Cells—DH5-α cells were transformed according to the instructions for the Canadian Life Technologies subcloning efficiency competent cells. Blue-white screen- ing was unnecessary since all the colonies tested contained the recombinant plasmid (efficiency = 100%). Colonies were picked from the agar plates using a sterile loop and transferred to tubes containing 10 ml of sterile LB (with 150 µg/ml ampicillin). The cells were allowed to grow overnight. Plasmid DNA was prepared from the cells using the small scale plasmid preparation protocol from Sambrook et al. (11). Restriction enzyme digests of the resulting plasmid DNA confirmed that Hb cDNA insert could be released as expected by digests using SstI/NotI or SstI/HindIII (data not shown).

Growth Conditions for Expression—Cells containing the recombi- tant and nonrecombinant (pUC 19) plasmids were grown for protein expression in sterile LB media containing 150 µg/ml ampicillin, 100 µg/ml 5-amino-levulinic acid, with or without 1 mM IPTG at 37 °C for 6–8 h. The bacterial cells were collected by centrifugation and frozen at −80 °C until used.

Electrophoresis and Determination of Subunit Molecular Mass—SDS-PAGE was performed using a Bio-Rad MiniProtein II gel appar-atus according to an established protocol (12). Final acrylamide mono- mer concentration in the 0.75-mm-thick gel was 22.5% for the separating gel and 4% (w/v) for the stacking gel. For the determination of subunit molecular mass, a plot of Rg versus log molecular mass was constructed using the following protein standards: BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and lysozyme (14.3 kDa).

Protein Concentration—Protein concentration was measured by the method of Bradford (13) using the Bio-Rad prepared reagent and BSA as standard.

Extraction and Purification of Recombinant Barley Hb—All proce-dures were performed at 4 °C, and all chromatographic separations used a Pharmacia FPLC system. All buffers were adjusted to pH and degassed at 20 °C. Washed cells (5 g, wet weight) were resuspended in 40 ml of extraction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA) and disrupted by three passes through a chilled French pressure cell at approximately 20,000 p.s.i. The bacterial cells were collected by centrifugation and frozen at −80 °C before mixing) because autoxidation reactions dominate at lower oxygen pressure. Apparent first order rates were calculated assuming a minimum and a maximum in the difference spectrum: HbCO minus HbO2. Measurements were limited to the range of ~250 µM dissolved oxygen (before mixing) because autoxidation reactions dominate at lower oxygen pressure. Apparent first order rates were calculated assuming a simple exponential decay using the Origins 4.1 program.

Carbon Monoxide Combination Rate—Solutions of ferrous barley Hb (5.8 µM heme in buffer containing a 3-fold molar excess of dithionite) was mixed rapidly with solutions of oxygen (250–1000 µM) in buffer, and the reaction was followed at 426 and 409 nm, respectively, a minimum and a maximum in the difference spectrum: HbO2 minus ferrous Hb. Measurements were limited to the range of ~250 µM dissolved oxygen (before mixing) because autoxidation reactions dominate at lower oxygen pressure. Apparent first order rates were calculated assuming a simple exponential decay using the Origins 4.1 program.

Carbon Monoxide Dissociation Rate—Solutions of ferrous barley Hb (5.8 µM heme in buffer containing a 3-fold molar excess of dithionite) were mixed rapidly with solutions of carbon monoxide (25–1000 µM in buffer), and the reaction was followed at 426 and 415 nm, respectively, a minimum and a maximum in the difference spectrum: HbCO minus ferrous Hb. Apparent first order rates were calculated assuming a simple exponential decay using the Origins 4.1 program.

Oxygen Dissociation Rate—A solution of barley Hb0 2 (3.5 µM Hb0 2; 27 µM free oxygen in buffer) was mixed rapidly with solutions of carbon monoxide (25–1000 µM in buffer), and the reaction was followed at 419 and 404 nm, respectively, a maximum and a minimum in the difference spectrum: HbCO minus ferrous Hb. To confirm the rate, the solution of HbO2 was mixed rapidly with 2 mM dithionite alone, and the reaction followed at 426 nm, a maximum in the difference spectrum: HbO2 minus ferrous Hb.

Carbon Monoxide Dissociation—Solutions of barley HbCO (3.2 µM HbCO, 5 µM free CO in buffer), prepared by equilibrating solutions of

1 The abbreviations used are: IPTG, isopropyl-β-D-thiogalactopyranoside; BSA, bovine serum albumin; HbO2, oxyhemoglobin; HbCO, carbon monoxide hemoglobin; 5-C, five coordinate; 6-C, six coordinate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.
HbO₂ first with CO at 1 atm and subsequently with CO in N₂ (P CO = 3.7 torr), were mixed rapidly with solutions of oxygen (680 and 1300 μM in buffer), and the reaction was followed at 419 nm, a maximum in the difference spectrum barley HbCO minus HbO₂. The rate was the same at lower HbCO concentrations (approximately 1 μM, n = 5). To confirm the findings, solutions of HbCO containing a slight excess of dithionite were mixed rapidly with a solution of NO (1,000 μM, in buffer). The reaction was followed at 418 nm, a minimum in the difference spectrum nitric oxide ferrous hemoglobin minus HbCO.

**Partition of Barley Hemoglobin between O₂ and CO**—A solution of barley HbO₂ (4.7 μM heme in buffer) was equilibrated at 1 atm total pressure with wet gas mixtures containing varying proportions of O₂ and CO. Autoxidation was minimal under this condition where the sum of the gas partial pressures was kept large. After equilibration at each gas composition was complete, optical spectra were acquired from 450 to 380 nm. Calculations were made from the sum of changes at 419 and 435.5 nm, respectively, a maximum, and an isosbestic point in the difference spectrum: barley HbCO minus HbO₂.

**RESULTS**

**Expression of Hb in E. coli**—Agar-LB plates of DH5α-cells transformed with the ligation mix produced many colonies. Cells grown from these colonies when compared with those containing the nonrecombinant pUC 19 were distinctly more red in color (not shown). Restriction digests of plasmid DNA preparations from these cells confirmed the existence of an insert that could be released by the expected restriction enzymes and had the expected molecular weight (approximately 800 base pairs, not shown). SDS-PAGE of crude protein extracts from these cells revealed a major band at 18.5 kDa that was found only in the lanes corresponding to the cells containing the recombinant plasmid (Fig. 1A). Addition of 1 mM IPTG to the bacterial suspension had no significant effect on the 18.5-kDa band (Fig. 1A). A 412 measurements of the crude extract (using a crude extract from nonrecombinant bacteria as a blank) gave an estimated concentration of extractable hemoglobin of 0.2 mg/g fresh weight compared with 4 mg/g fresh weight extractable total soluble protein. From these figures and visual analysis of SDS-PAGE (Fig. 1A), the fusion protein appears to be approximately 5% of the total soluble bacterial protein.

**Purification of Recombinant Barley Hb**—The purification of barley Hb is shown in Table I. Recombinant barley Hb was purified 16.2-fold to a final purity of over 97% as assessed by A₄₁₂ measurements with a yield of 5%. Purified recombinant Hb was over 95% pure as assessed by SDS-PAGE (Fig. 1B).

**Characterization of Barley Hb**—The recombinant Hb has an expected N-terminal sequence of Met-Ile-Thr-Pro-Ser-Leu-Ala-Ala-Glu as compared with the native Hb N-terminal sequence of Met-Ser-Ala-Ala-Glu and therefore has five additional amino acids. SDS-PAGE of the final purified recombinant protein confirms that the expressed Hb has the expected molecular mass (Fig. 1B). Native molecular mass as assessed by size exclusion chromatography on Superose 12 was determined to be 40 kDa (± 4 kDa (n = 3)). This suggests that barley hemoglobin is a homodimer.

The amino acid composition of the native and recombinant hemoglobin is shown in Table II, except for glycine which gave unreliable results probably due to the presence of glycine in the electrophoretic transfer buffer. The observed amino acid composition of the native and recombinant Hb were, within the error of the determination, generally in agreement with the expected, deduced composition, based on nucleotide sequence data. Methionine, aspartate, glutamate, and lysine were exceptions. Methionine is noted for being problematic in amino acid determinations. When asparagine and glutamine levels are expressed in combination, the results (Table II) are in agreement with the expected levels. The differences between expected and observed values for lysine are not readily explainable, except to suggest that the differences could have resulted from modifications during the analysis process. It is notable, however, that the levels of lysine are in agreement between the native and recombinant barley hemoglobin. These results suggest that the native and recombinant proteins differ only in the additional five amino acids at the N-terminal end of the recombinant protein.

**Optical Spectra**—The spectral properties of recombinant barley Hb are reported in Table III, and optical spectra are presented in Figs. 2 and 3. The spectra are essentially invariant from pH 6.0 to pH 9.0. To be certain that the spectrum of barley Hb is shown in Table III.
Reactions of Barley Hemoglobin with Ligands

A single homogeneous kinetic event ($k = 0.0272 \text{ s}^{-1}$; $n = 5$), independent of both CO and dithionite concentration, was observed in the replacement of bound oxygen by carbon monoxide in the presence of dithionite. A single but inhomogeneous rate ($k = 0.0243 \text{ s}^{-1}$; $n = 2$) was observed in the absence of CO. To eliminate the possibility that dithionite was reacting with HbO$_2$ without prior dissociation of bound oxygen, the reaction was followed in the presence of CO alone. The observed rate ($0.013 \text{ s}^{-1}$ at 1000 mM CO) serves to substantiate the rates observed in the presence of dithionite but is about 2-fold less and approximately proportional to CO concentration. Evidently CO does not compete effectively with dissolved oxygen in the solution.

Carbon Monoxide Dissociation—A single homogeneous rate ($k = 1.10 \times 10^{-3} \text{ s}^{-1}$; $n = 3$) was observed in replacement of bound CO by O$_2$. In confirmation, using a less pure sample of HbCO, a single but inhomogeneous rate ($1.2 \times 10^{-3} \text{ s}^{-1}$; $n = 2$) was observed in the replacement of bound CO by O$_2$ and an inhomogeneous rate ($1.4 \times 10^{-3} \text{ s}^{-1}$; $n = 5$) in replacement of bound CO by NO.

Partition of Ferrous Hemoglobin between Oxygen and Carbon Monoxide—A plot of the ratio HbO$_2$/HbCO against the ratio oxygen partial pressure/carbon monoxide partial pressure was linear over the range examined (Fig. 5). The partition coefficient, $M$, given by the reciprocal of the slope of this relation, is $M = 1.48$.

Second Order Oxygen Combination Rate Constant—This was calculated from the value of the partition coefficient and the three already established rate constants. The partition coefficient, $M$, expresses the relative affinities of ferrous Hb for O$_2$ and CO: $M = k_{cp}/k_{dp}$, where $K_{cp}$ and $L_{dp}$ are the binding constants for O$_2$ and CO, respectively. $K_{dp}$ and $L_{dp}$ in turn express the ratios of the respective dissociation and combination rate constants: $K_{dp} = k'_{off,O2}/k'_{on,O2}$ and $L_{dp} = k'_{off,CO}/k'_{on,CO}$. Entering the known values into the expression for $M$, initial concentration of CO exceeds the Hb concentration by 4.3- and 8.6-fold, respectively. Second order combination rate constants were approximated as the observed first order rate divided by the ligand concentration. The average value at these two CO concentrations is $k = 0.57 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ($n = 10$).

Overall Oxygen and Carbon Monoxide Combination Rates—Single rates following apparent first order kinetics were seen at all ligand concentrations. The rates with each ligand were independent of the observing wavelength. Above 200 mM ligand concentration (after mixing), the rates of O$_2$ and CO combination are similar and independent of ligand concentration (Fig. 4). The CO combination rates decline monotonically toward zero at zero ligand concentration. The maximum combination rate for O$_2$ ($43 \text{ s}^{-1}$), determined graphically from Fig. 4, is close to that found for CO ($41 \text{ s}^{-1}$).

Second Order Carbon Monoxide Combination Rate Constant—At the two lowest CO concentrations examined, 12.6 and 25.2 mM CO (after mixing), the observed combination rate becomes small relative to the maximum rate (Fig. 4), and the
we obtain an approximate numerical value for the second order rate constant for combination of O$_2$ with 5-C ferrous Hb ($k_{on,O2} = 9.5 \times 10^6$ M$^{-1}$ s$^{-1}$).

Characterization of Native Hemoglobin—Native barley Hb was purified from seeds germinated on Petri dishes for 3 days.$^2$ The native barley Hb was a dimer with subunit molecular mass approximately 18 kDa. The optical spectrum of the native Hb is indistinguishable from the recombinant protein (Table III, Fig. 1). The oxygen dissociation rate for the native Hb was 0.022 s$^{-1}$ which is virtually identical to the recombinant protein$^3$ (Table IV).

DISCUSSION

Expression and Purification of Barley Hemoglobin—Recombinant barley hemoglobin has been purified to homogeneity and was found to be a homodimer with subunit molecular mass of 18.5 kDa, in contrast to most symbiotic hemoglobins that are monomers (3, 10). The distinct red color of the recombinant bacteria and the purified protein, as well as its absorbance spectra, is strong evidence that the bacteria can properly produce a monocot globin protein capable of binding heme and constitute definitive evidence that the cloned Hb cDNA does in fact represent a Hb gene.

Properties and Function—The isolated, recombinant barley hemoglobin displays an optical spectrum in the visible and
Soret regions which is very similar to those of many oxygenated heme proteins (Table III, Fig. 2). An equilibrium oxygen affinity cannot be demonstrated; deoxygenation generates a mixture of species in which the ferric protein predominates. However, reversible replacement of bound oxygen by carbon monoxide to generate a species with the unmistakable and characteristic spectrum of carbon monoxide hemoglobin (Table III, Fig. 2) establishes the identity of the putative oxygenated species.

Ferric barley hemoglobin exhibits an optical spectrum (Table III, Fig. 3) reminiscent of many low spin 6-C ferric heme proteins such as ferric myoglobin cyanide, isocyanide, sulfide, or hydroxide (15). The optical spectrum is distinct from that expected of a 5-C species (16). The ferric oxidation state is confirmed by dithionite reduction to the low spin 6-C ferric protein.

Ferrous barley hemoglobin displays an optical spectrum with prominent maxima in the visible region at 529 and 563 nm (Fig. 3). This spectral pattern is strongly reminiscent of a number of 6-C low spin ferrous derivatives with oxygen, nitrogen, or sulfurous ligands in the distal ligand position. These include ferrous hemochromogens, ferrous protoheme cytochromes including cytochrome c, ferrous myoglobin or horseradish peroxidase cyanides (17), ferrous myoglobin (18), or leghemoglobin (19) nicotinates, and ferrous heme proteins in which the ligand atom immediately adjacent to the heme iron is oxygen (17).

Ligation of a distal nitrogenous residue to the heme iron to generate 6-C species has precedents in other hemoglobins. For instance, ferric bis-histidyl kidney and soybean leghemoglobins are in temperature-dependent equilibria with their aquoferrous forms (20). More dramatically, oxygenated gial hemoglobins of instance, ferric bis-histidyl kidney and soybean leghemoglobins generate 6-C species has precedents in other hemoglobins. For ferrous myoglobin (18), or leghemoglobin (19) nicotinates, and ferrous myoglobin or horseradish peroxidase cyanides (17), ferrous myoglobin (18), or leghemoglobin (19) nicotinates, and ferrous heme proteins in which the ligand atom immediately adjacent to the heme iron is oxygen (17).

Reaction 1:

\[ k_{1} \]

6-C ferrous Hb \rightleftharpoons 5-C ferrous hemoglobin

**REACTION 1**

Very recently Lamb et al. (25) have prepared a derivative of low spin ferrous sperm whale myoglobin with a water molecule in the distal position at 20 K. This species exhibits an optical spectrum very similar to that of ferrous barley hemoglobin, with a Soret maximum at 428 nm and conspicuously split 4 bands near 527 and 555 nm and the other giving rise to the 535- and 563-nm paired bands (18). Sequence alignment suggests that histidine occupies the position distal to the heme iron (1), but it remains entirely possible that other nearby residues may contribute the actual ligand.

At the largest attainable concentrations of attacking ligand (0.5 atm, 504 \( \mu \)M CO, 675 \( \mu \)M O2, after mixing), the rates of combination of CO or O2 with 5-C ferrous Hb (Reaction 2) may exceed the rate of formation of 5-C from 6-C ferrous Hb (Reaction 1). In this event, dissociation of the sixth ligand (Reaction 1) will limit the overall combination rate (k\(_{+3}\) of Reaction 3). This kinetic model indeed fits the facts. At high ligand concentration, the rates of combination of oxygen and carbon monoxide with ferrous barley hemoglobin each become independent of ligand concentration and, within the constraints of the experiment, are essentially the same (k\(_{22}\) = 43 s\(^{-1}\), k\(_{CO}\) = 41 s\(^{-1}\)) (Fig. 4). This implies a common rate-limiting step, which, we suggest, is conversion of 6-C to a 5-C ferrous Hb (see Reaction 1).

Reaction 2:

\[ k_{2} \]

5-C ferrous Hb + ligand \rightleftharpoons 5-C ferrous Hb

**REACTION 2**

4 D. C. Lamb, personal communication.
Combining, we obtain the overall reaction,

\[ k_3 \quad 6\text{-C ferrous Hb} + \text{ligand} \rightarrow \text{Hb-L} \quad k_3 \]

**Reaction 3**

The numerical value of the rate of 6-C to 5-C ferrous Hb conversion, \( k_{-1} \) of Reaction 1 is given by the maximum overall rate of oxygen or CO combination, \( k = 43 \text{ s}^{-1} \) or \( k = 41 \text{ s}^{-1} \), respectively. The rate of the converse reaction (\( k_{-1} \) of Reaction 1) was not determined. Since \( k_{-1} \) is rate-limiting at high ligand concentration, \( k_{-1} \approx k_{-1} \cdot k_{-1} \). The second order rate constant for combination of ligand (O2 or CO) with 5-C ferrous hemoglobin (\( k_{-2} \) of Reaction 2) and the two ligand dissociation rate constants (\( k_{-3} \) of Reaction 2) are given in Table II.

The rate of deoxygenation of barley HbO2 is among the slowest known, exceeded only by Ascaris Hb (Table III). Slow oxygen dissociation implies stabilization of the bound ligand, commonly by hydrogen bond formation from a nearby or distal residue to the bound oxygen, as in soybean leghemoglobin (Table III; Ref. 24). In the absence of such stabilization, dissociation is fast, exemplified by Mb (His(E7) \( \rightarrow \) Gly), Table IV). Very slow oxygen dissociation may imply further stabilization, for instance in Ascaris Hb (Table IV) by interaction of the bound oxygen with both the distal residue and a tyrosine in position B10 (26–29). Phenylalanine introduced into position B10 may also interact, decreasing the dissociation rate about 10-fold (30). Sequence alignment (1) places phenylalanine at position B10 may also interact, decreasing the dissociation rate about 10-fold (30). Sequence alignment (1) places phenylalanine at position B10 may also interact, decreasing the dissociation rate about 10-fold (30). Sequence alignment (1) places phenylalanine at position B10 of barley hemoglobin. Indeed all plant hemoglobins have either phenylalanine or tyrosine at this position (6), but none appear to interact with the bound ligand.

**Parasponia** Hb, a nodule hemoglobin encoded by a gene in the nonsymbiotic Hb family, invites comparison with barley nonsymbiotic Hb. Both *Parasponia* and barley Hb amino acid sequences fall within the cluster of nonsymbiotic hemoglobins (6). Both are homodimers (31). Yet *Parasponia* hemoglobin, isolated from the root nodule, displays chemical reactivities characteristic of symbiotic hemoglobins (24, 31), which are entirely different from those described here for barley Hb. We consider that subtle and precise local structural changes near the heme may dictate large differences in chemical behavior with conservation of the globin fold and of the particular structural arrangement dictated by the nonsymbiotic amino acid sequence.

Extraordinarily slow dissociation of oxygen from barley HbO2 (\( t_{1/2} = 25 \text{ s} \)) would appear to rule out reaction paths involving reversible oxygen dissociation. This, in itself, does not rule out facilitation of oxygen diffusion because ligand dissociation from *Ascaris* myoglobin and from two cytoplasmic hemoglobins in the gill of the clam *Lucina*, which are believed to facilitate diffusion of their ligands, is also very slow (14, 32). Alternatively, the 6-coordinate conformation of ferrous and ferric barley Hb may suggest a role of barley hemoglobin in electron transfer, possibly to a bound oxygen molecule.

Small amounts of the native Hb were purified, and its subunit structure, optical spectrum, and oxygen dissociation constant were compared with those of the recombinant protein. Similarly, the amino acid compositions of the native and the recombinant Hb were in agreement with their expected deduced compositions based on the nucleotide sequences. All of these properties of the native protein were indistinguishable from those of the recombinant protein, indicating that the characteristics of the recombinant protein adequately reflect those of native barley hemoglobin.

Acknowledgments—We are grateful to Drs. Brian Fristensky, Joel Friedman, Cyril A. Appleby, and Alberto Boffi for helpful and stimulating discussions and to Doug Durmin for technical assistance and to Dr. A. W. MacGregor for the use of his facilities.

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