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

OPTICAL SPECTRA AND REACTIONS WITH GASEOUS LIGANDS*

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Stephen M. G. Duff‡, Jonathan B. Wittenberg§, and Robert D. Hill‡

From the §Department of Physiology & Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461 and the ‡Department of Plant Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

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 actinomycete *Frankia* and variety of non-leguminous woody dicotyledonous plants. In a unique instance, the bacterium *Rhizobium* nodulates a non-legume *Parasponia andersonii* (3). Although symbiotic hemoglobins show large differences in their geminate 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 10^5 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 nM, 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 Hb 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 Canadian Life Technologies. GeneClean II Kit was from Bio-Can Scientific. 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 (1) between the restriction sites *Sst*I and *Xba*I, was used as the starting material. The proce-

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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 reading frame.

The plasmid containing the insert (approximately 100 μg) was digested for 6 h with *Sst*II (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, ATCGCCGG) was then phosphorylated and allowed to anneal with the 14-mer (single-stranded adapter, AGCTTAGCGGCCGC) to form a linker-adapter containing an *Sst*II site at one end, *Hin*dIII site at the other end, and a *Not*I site in the middle. The adapter was then ligated to the insert end of the *Sst*II-digested plasmid. The plasmid was then digested with *Sst*I releasing the insert cDNA (with the linker-adapter ligated to it forming a *Hin*dIII 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 *Sst*I and *Hin*dIII 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 screening 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 *Sst*I/*Not*I, *Sst*I/*Sst*II, or *Sst*I/*Hin*dIII (data not shown).

Growth Conditions for Expression—Cells containing the recombinant and nonrecombinant (pUC 19) plasmids were grown for protein expression in sterile LB media containing 150 μg/ml ampicillin, 100 μg/ml δ-aminolevulinic acid, with or without 1 mM IPTG[1] 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 Miniprotean II gel apparatus according to an established protocol (12). Final acrylamide monomer concentration in the 0.75-mm thick slab gels was 15% (w/v) for the separating gel and 4% (w/v) for the stacking gel. For the determination of subunit molecular mass, a plot of R_F *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 Determination—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 procedures 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% sucrose (w/v), 1 mM dithiothreitol, 1 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml chymostatin, 10 μg/ml E-64) and disrupted by three passes through a chilled French pressure cell at approximately 20,000 p.s.i. The lysate was clarified by centrifugation at 27,000 × *g* for 10 min. The supernatant fluid was diluted to 50 ml with extraction buffer and fractionated with polyethylene glycol 8000. The red colored 10–22% polyethylene glycol pellet was redissolved in 30 ml of buffer A (50 mM Tris-HCl, pH 8.5. 1 mM dithiothreitol, 1 mM EDTA) and the clarified solution applied at a rate of 1 ml/min to a column (1.5 × 6 cm) of Q-Sepharose (Pharmacia Biotech Inc.) preequilibrated with buffer A. After thorough washing at 1 ml/min, the protein was eluted at the same flow rate with a 100-ml linear gradient of 0–500 mM KCl in buffer A. The fractions eluting around 150–200 mM KCl contained most of the red color and were pooled and diluted approximately 2.5–3-fold with buffer A. The pooled, diluted Hb sample was then applied at a flow rate of 0.5 ml/min to a Mono Q HR 5/5 column equilibrated with buffer A. The Hb was then eluted at a flow rate of 1 ml/min with a 50-ml linear gradient of 0–500 mM KCl in buffer A. The fractions eluting around 160 mM KCL were pooled. Ammonium sulfate was added to the pooled sample to a final concentration of 30% (w/v) and dissolved. The sample was then loaded onto a prepacked phenyl-Sepharose column equilibrated with buffer A containing 30% (w/v) ammonium sulfate. Hb was then eluted at a flow rate of 1 ml/min with a 50-ml linear gradient of 30–0% (w/v) ammonium sulfate in buffer A. The fractions eluting from the phenyl-Sepharose column were analyzed by SDS-PAGE, and the most pure fractions were pooled and concentrated to a final volume of approximately 200 μl and buffer exchanged into PBS (40 mM KH_2PO_4/K_2HPO_4, pH 7.0, 150 mM NaCl) using a Centricon 10 concentrator. The purified Hb was then either immediately used for analysis or stored at −80 °C until needed.

Molecular Mass of Recombinant Hemoglobin—The native molecular mass of recombinant barley root Hb was determined by gel filtration on a prepacked Superose 12 HR 10/30 column using 0.2-ml sample volumes and 50 mM Tris-HCl, pH 9.0, 150 mM KCl as column buffer. Fractions (0.5 ml) were collected with a flow rate of 0.2 ml/min and assayed for A_{280} and A_{412} (absorbance maxima of barley HbO_2). The native molecular mass of the fusion protein was determined from a plot of K_{av} (partition coefficient) *versus* log molecular mass for the following protein standards: alcohol dehydrogenase (150 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and ribonuclease (14 kDa).

Amino Acid Analysis—Purified native and recombinant hemoglobin underwent electrophoresis using the Bio-Rad minigel system (Ref. 12, see above) and was then transferred using the Bio-Rad system to polyvinylidene difluoride membrane (Bio-Rad). The membrane was then stained using 0.01% Coomassie Blue, 40% (v/v) methanol, and destained in 40–50% methanol until the hemoglobin band was apparent. The Hb band was excised and subjected to amino acid analysis using an Applied Biosystems Model 420 amino acid derivatizer system.

Optical Spectra—These were acquired using a modified Cary model 17 recording spectrophotometer (Aviv Associates, Lakewood, NJ) equipped with an Aviv data acquisition and analysis system.

Hemoglobin Concentration—Total heme was determined using a pyridine-hemochromogen assay (14). Hemoglobin concentration was determined by Bradford protein assay or by A_{412} measurements.

Buffer for Ligand Binding Experiments—50 mM potassium phosphate buffer, pH 7.40, containing 0.5 mM EDTA was used throughout. The temperature was 20 °C.

Ligand Reaction Rates—These were measured using an Hi-Tech model SF 61 (Salisbury, UK) stopped-flow apparatus interfaced to an OLIS Data Acquisition/Computation System (On-Line Instrument Systems, Bogart, GA). Rates were computed using either the Olis system or Origins 4.1 (Microcal Software Northampton, MA). Most kinetic experiments used Hb purified by chromatography on Mono Q HR only, omitting the final hydrophobic interaction chromatography step.

Oxygen Combination Rate—A solution 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: HbO_2 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 single 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) were mixed rapidly with solutions of carbon monoxide (25–1000 μM in buffer), and the reaction was followed at 428 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 single exponential decay using the Origins 4.1 program.

Oxygen Dissociation Rate—A solution of barley $Hb0_2$ (3.5 μM HbO_2; 27 μM free oxygen in buffer) was mixed rapidly with solutions of carbon monoxide (250–1000 μM in buffer containing 0–4 mM dithionite), and the reaction was followed at 419 and 404 nm, respectively, a maximum and a minimum in the difference spectrum: HbCO minus HbO_2. To confirm the rate, the solution of HbO_2 was mixed rapidly with 2 mM dithionite alone, and the reaction followed at 426 nm, a maximum in the difference spectrum: HbO_2 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; HbO_2, oxyhemoglobin; HbCO, carbon monoxide hemoglobin; 5-C, five coordinate; 6-C, six coordinate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

HbO_2 first with CO at 1 atm and subsequently with CO in N_2 (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 HbO_2. The rate was the same at lower HbCO concentration (approximately 1 μM, n = 3). 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 O_2 and CO—A solution of barley HbO_2 (4.7 μM heme in buffer) was equilibrated at 1 atm total pressure with wet gas mixtures containing varying proportions of O_2 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 HbO_2.

RESULTS

Expression of Hb in E. coli—Agar-LB plates of DH5-α 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. 1*A*). Addition of 1 mM IPTG to the bacterial suspension had no significant effect on the 18.5-kDa band (Fig. 1*A*). 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. 1*A*), the fusion protein appears to be approximately 5% of the total soluble bacterial protein.

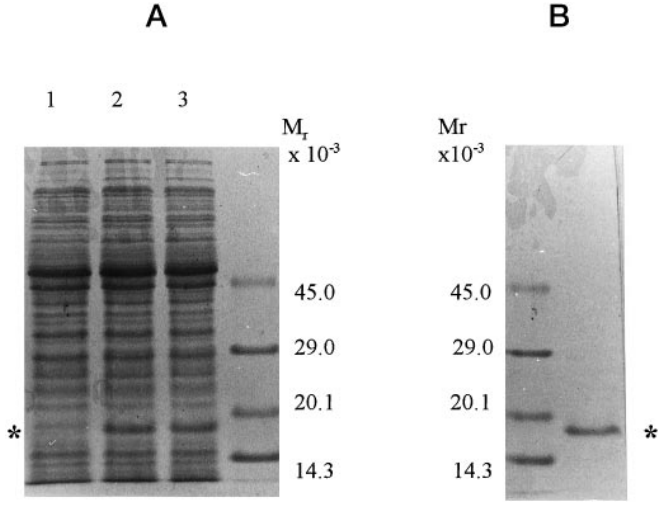

FIG. 1. **SDS-PAGE of recombinant Hb.** *A,* SDS-PAGE of *E. coli* crude protein extracts showing recombinant barley root Hb fusion protein. The *right-hand lane* contains 10 μg of various protein standards described under "Materials and Methods"; *lane 1*, 60 μg of crude extract from *E. coli* transformed with pUC 19 plasmid; *lane 2*, 60 μg of crude extract from *E. coli* transformed with the recombinant plasmid as described under "Materials and Methods"; *lane 3*, as in *lane 2* except *E. coli* was grown in 1 mM IPTG; *B,* SDS-PAGE of purified recombinant barley root Hb fusion protein. The *right-hand lane* contains 2 μg of the final preparation, whereas the *left lane* contains 5 μg of the various protein standards described under "Materials and Methods."

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 A_{412} measurements with a yield of 5%. Purified recombinant Hb was over 95% pure as assessed by SDS-PAGE (Fig. 1*B*).

Characterization of Barley Hb—The recombinant Hb has an expected N-terminal sequence of Met-Ile-Thr-Pro-Ser-Leu-Ala-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. 1*B*). Native molecular mass as assessed by size exclusion chromatography on Superose 12 was determined to be 40 kDa ($\pm$4 kDa (n = 3)). This suggests that barley hemoglobin is a homodimer.

The amino acid composition of the native[2] 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 asparate + asparagine and glutamate + 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

[2] Germination of barley seeds, antibody preparation, affinity purification of Hb antibodies, preparation of an immunoaffinity column, and immunoaffinity chromatography of barley Hb will be described in a future article (manuscript in preparation).

TABLE I
Purification of recombinant barley hemoglobin

Fraction	Protein	Hb[a]	Hb	F.P.[b]	Yield
	mg	*mg*	*%*		*%*
Crude extract	249	15.1	6.0	1.0	100
10–22% PEG precipitation	112	12.5	11.2	1.9	83
Q-Sepharose chromatography	12	8.9	75.0	12.5	58
Mono-Q FPLC	6	5.2	86.7	14.2	34
Phenyl-Sepharose FPLC	1.3	1.2	98.0	16.2	5

[a] Amount of Hb determined by A_{412} measurements and a standard curve.
[b] Fold purification.

TABLE II
Amino acid composition of native and recombinant barley hemoglobin

Amounts of each amino acid relative to alanine as 1.00 (pmol).

Amino acid	Native hemoglobin		Recombinant hemoglobin	
	Expected	Observed	Expected	Observed
Met	0.31	0.27	0.30	0.21
Ser	0.31	0.34	0.30	0.35
Asp	0.28 (0.42)[a]	0.44	0.26 (0.41)[a]	0.44
Gly	0.41	Unreliable[b]	0.39	Unreliable[b]
Ala	1.00	1.00	1.00	1.00
Glu	0.69 (0.86)[c]	0.82	0.66 (0.83)[c]	0.83
Val	0.50	0.52	0.49	0.49
Phe	0.41	0.42	0.39	0.39
Lys	0.63	0.51	0.61	0.48
Ile	0.28	0.29	0.29	0.28
Asn	0.14	ND[d]	0.13	ND
Arg	0.36	0.36	0.35	0.36
Pro	0.32	0.36	0.34	0.34
Thr	0.41	0.37	0.44	0.39
Leu	0.72	0.72	0.74	0.75
Trp	0.09	ND	0.09	ND
His	0.13	0.13	0.13	0.09
Cys	0.04	0.02	0.04	0.02
Tyr	0.09	0.09	0.09	0.10
Gln	0.18	ND	0.04	ND

[a] Expected aspartate + asparagine.
[b] Glycine present in the electrophoresis buffer (12).
[c] Expected glutamate + glutamine.
[d] ND, not determined.

TABLE III
Spectral properties of recombinant barley hemoglobin

Derivative	λ_{max}	ϵ_{mM}
Ferrous Hb	425	190
	529, 535	15.0, 14.5
	555, 563	20.5, 21.2
HbO_2	412	149
	540	17.3
	576	17.3
HbCO	417	202
	537	16.6
	567	16.4
Ferric Hb	411	141
	534	22
	565	

deoxy-Hb was independent of the route by which the compound was formed, deoxy-Hb was prepared in three ways: 1) reaction of ferric Hb with dithionite, 2) reaction of HbO_2 with large excess of dithionite, and 3) removal of carbon monoxide from a solution of HbCO sparged with inert gas (dithionite was present to prevent ferric Hb formation). The optical spectrum was in each case the same. As discussed below, spectra of the ferric and deoxy ferrous forms suggest that these are low spin six-coordinate (6-C) species.

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 μM 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 s^{-1}), determined graphically from Fig. 4, is close to that found for CO (41 s^{-1}).

Second Order Carbon Monoxide Combination Rate Constant—At the two lowest CO concentrations examined, 12.6 and 25.2 μM CO (after mixing), the observed combination rate becomes small relative to the maximum rate (Fig. 4), and the 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 $k = 0.57 \times 10^6$ M^{-1} s^{-1} ($n = 10$).

Oxygen Dissociation—A single homogeneous kinetic event ($k = 0.0272$ 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 ($k = 0.0243$ 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 s^{-1} at 1000 μM 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 ($k = 1.10 \times 10^{-3}$ 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 (1.2×10^{-3} s^{-1}; $n = 2$) was observed in the replacement of bound CO by O_2 and an inhomogeneous rate (1.4×10^{-3} 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: $M = K_D/L_D$, where K_D and L_D are the equilibrium binding constants for O_2 and CO, respectively. K_D and L_D in turn express the ratios of the respective dissociation and combination rate constants: $K_D = k'_{off,O2}/k_{on,O2}$ and $L_D = k_{off,CO}/k'_{on,CO}$. Entering the known values into the expression for M,

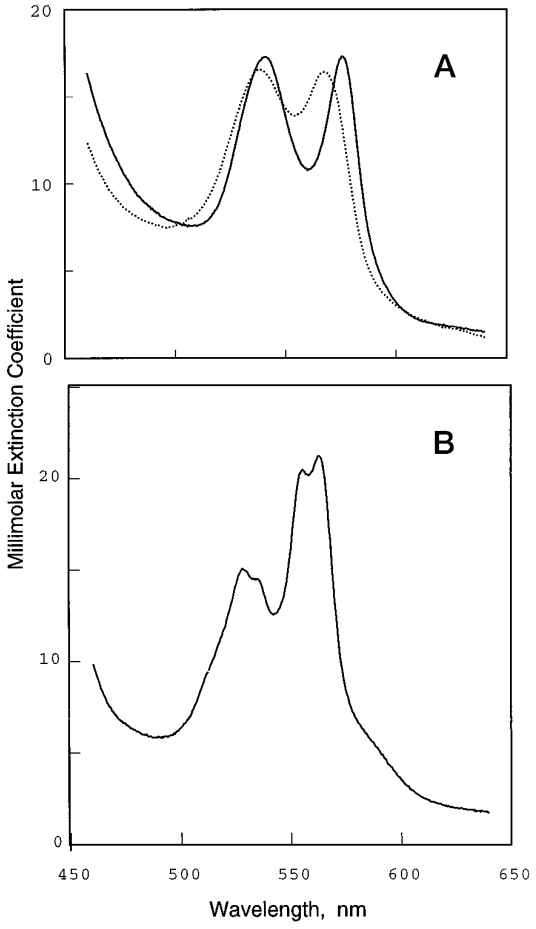

FIG. 2. **Optical spectra of ferrous barley hemoglobin derivatives in the visible region.** *A*, *solid line*, oxyhemoglobin; *dotted line*, carbon monoxide hemoglobin. *B*, ferrous (deoxy) barley hemoglobin. This spectrum suggests that the heme iron of the deoxy ferrous species is low spin and 6-coordinate.

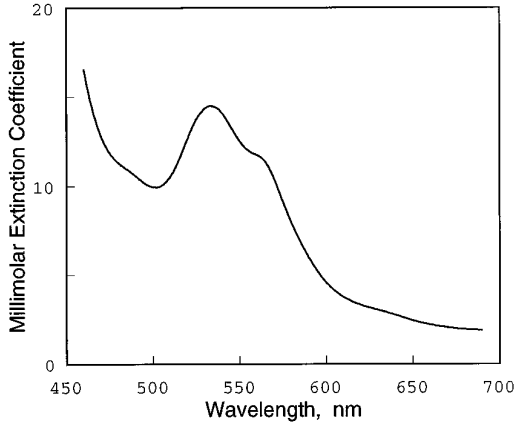

FIG. 3. **Optical spectrum of ferric barley hemoglobin in the visible region.** This spectrum suggests that the heme iron of the ferric species is low spin.

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}

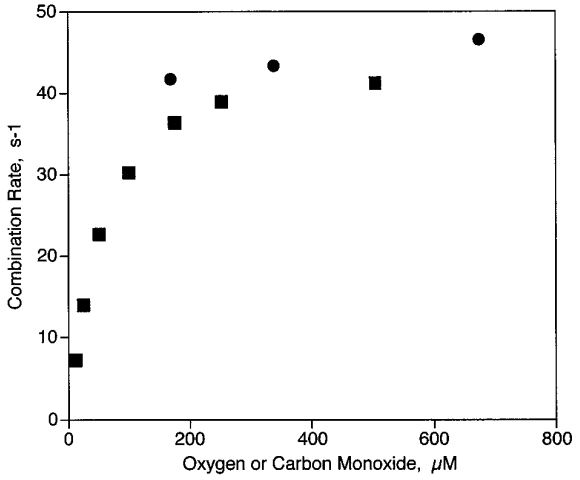

FIG. 4. **Rates of combination of oxygen and carbon monoxide with ferrous barley hemoglobin as a function of ligand concentration (after mixing).** *Solid circles*, oxygen; *solid squares*, carbon monoxide. Maximum rates are 43 and 41 s^{-1} for oxygen and carbon monoxide, respectively. For each data point, $n = 4$–9, and the standard error of the mean does not exceed 2% of the reported value.

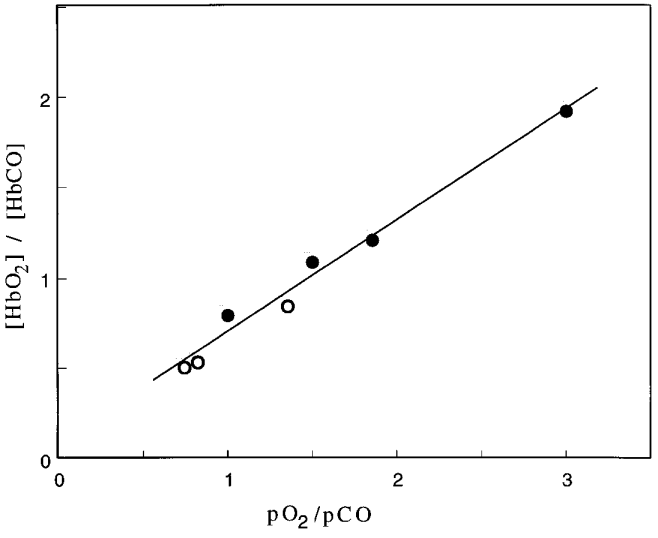

FIG. 5. **Partition of barley hemoglobin between oxygen and carbon monoxide.** Different symbols represent separate experiments. The partition coefficient, $M = 1.48$, is given by the slope of the line determined by a least squares fit.

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

[3] Subunit structure, optical spectrum, and oxygen dissociation constant of the native Hb were determined exactly as for the recombinant Hb.

TABLE IV

Kinetics and equilibrium constants for the reactions of ferrous barley hemoglobin with oxygen and carbon monoxide compared with those of other proteins

All data were obtained at pH 7.5 and 20 °C.

Protein	Oxygen			Carbon monoxide			M
	k'_{on}	k_{off}	K_D (=k:k')	I'_{on}	I_{off}	L_D (=I:I′)	K_D/L_D
	$\times 10^{-6}\ M^{-1}\ s^{-1}$	s^{-1}	nM	$\times 10^{-6}\ M^{-1}\ s^{-1}$	s^{-1}	nM	
Barley Hb	9.5	0.0272	2.86	0.57	0.0011	1.93	1.48
Soybean Lb[a]	120	5.6	48	13	0.0078	0.62	78[a]
Parasponia Hb[a]	165	15	89	14	0.019	1.4	64
Sperm whale Mb[b]	14	12	857	0.51	0.019	37	23[b]
Mb (His(E7)-Gly)[b]	140	1,600	11,400	5.8	0.038	6.6	1,700[b]
Ascaris Hb[c]	1.5	0.0041	2.7	17	0.018	1.1	2.5[c]

[a] Data of Gibson *et al.* (5).
[b] Data of Springer *et al.* (26).
[c] Data of Gibson and Smith (32).

Soret regions which is very similar to those of many oxygenated hemeproteins (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 hemeproteins 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 ferrous 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 oxygenous, nitrogenous, or sulfurous ligands in the distal ligand position. These include ferrous hemochromogens, ferrous protoheme cytochromes including cytochrome *b*, ferrous cytochrome *c*, ferrous myoglobin or horseradish peroxidase cyanides (17), ferrous myoglobin (18), or leghemoglobin (19) nicotinates, and ferrous hemeproteins 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 aquoferric forms (20). More dramatically, oxygenated glial hemoglobins of the nerves of the clams *Spisula* (21, 22) and *Tellina* (23), both *in vivo* and *in vitro*, are in freely reversible equilibrium with oxygen and low spin 6-C species. Normal deoxy derivatives are not seen.

The α and β bands of the ferrous barley hemoglobin optical spectrum are each double, with well resolved maxima at 555 and 563 nm and 527 and 535 nm, respectively (Fig. 3). The Soret band at 426 nm, however, is narrow with the half-bandwidth (20 nm) expected for a single species. Such four-banded visible spectra have been encountered in the ferrous hemochromogens formed reversibly by reaction of mammalian myoglobins with moderate concentrations of the nitrogenous ligands hydrazine, pyridine, β-picoline, and especially, nicotinic acid (pyridine 3-carboxylic acid) (18). The nicotinic acid-myoglobin complex contains one molecule of nicotinic acid per atom of heme iron (18), and the pyridine ring nitrogen is ligated to the sixth ligand position of the heme iron atom of both ferrous and ferric leghemoglobin nicotinates (20, 24). Accordingly, a simple but not unique explanation of the four-banded spectrum may be that a nitrogenous ligand, most probably the side chain of an amino acid of the backbone polypeptide chain, may bind to the heme in two different, slowly interconverting conformations: one conformer giving rise to absorbance 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.

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 α and β bands near 560 and 530 nm, respectively.[4] This suggests that ferrous barley hemoglobin may be a low spin 6-C species with water (or similar oxygen-containing ligand) in the sixth coordination position.

The 6-C nature of the ferrous derivative has interesting consequences for the reactions of barley hemoglobin with ligands. *A priori*, one would expect a leaving ligand to leave behind a 5-C species and only subsequently could a ligand from the distal pocket attack the heme iron atom to generate a 6-C form. Conversely, an attacking ligand would be expected to react only with the 5-C form, implying prior dissociation of the sixth ligand from the 6-C iron atom.

At the largest attainable concentrations of attacking ligand (0.5 atm, 504 μM CO, 675 μM O_2, after mixing), the rates of combination of CO or O_2 with 5-C ferrous Hb (Reaction 2) may exceed the rate of formation of 5-C from 6-C ferrous Hb (k_{+1} of 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_{O2} = 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).

$$\text{6-C ferrous Hb} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{5-C ferrous hemoglobin}$$

REACTION 1

$$\text{5-C ferrous Hb + ligand} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{Hb ligand}$$

REACTION 2

[4] D. C. Lamb, personal communication.

Combining, we obtain the overall reaction,

$$\text{6-C ferrous Hb + ligand} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \text{Hb-L}$$

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, $k = 43\ s^{-1}$ or $k = 41\ s^{-1}$, 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, $k_{+3} = k_{+1}$. The second order rate constant for combination of ligand (O_2 or CO) with 5-C ferrous hemoglobin (k_{+2} of Reaction 2) and the two ligand dissociation rate constants (k_{-2} of Reaction 2) are given in Table II.

The rate of deoxygenation of barley HbO_2 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 or whale myoglobin (Table III; Ref. 24). In the absence of such stabilization, dissociation is fast, exemplified by Mb (His(E7) → 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 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 HbO_2 ($t_{½}$ = 25 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.

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