Recombinant Human Erythrocyte Cytochrome b_5[†]

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ABSTRACT: The gene encoding the human erythrocyte form of cytochrome b_5 (97 residues in length) has been prepared by mutagenesis of an expression vector encoding lipase-solubilized bovine liver microsomal cytochrome b_5 (93 residues in length) (Funk et al., 1990). Efficient expression of this gene in *Escherichia coli* has provided the first opportunity to obtain this protein in quantities sufficient for physical and functional characterization. Comparison of the erythrocytic cytochrome with the trypsin-solubilized bovine liver cytochrome b_5 by potentiometric titration indicates that the principal electrostatic difference between the two proteins results from two additional His residues present in the human erythrocytic protein. The midpoint reduction potential of this protein determined by direct electrochemistry is -9 ± 2 mV vs SHE at pH 7.0 (μ = 0.10 M, 25.0 °C), and this value varies with pH in a fashion that is consistent with the presence of a single ionizable group that changes pK_a from 6.0 ± 0.1 in the ferricytochrome to 6.3 ± 0.1 in the ferrocytochrome with $\Delta H° = -3.2 \pm 0.1$ kcal/mol and $\Delta S° = -11.5 \pm 0.3$ eu (pH 7.0, μ = 0.10). The 1D ^{1}H NMR spectrum of the erythrocytic ferricytochrome indicates that 90% of the protein binds heme in the "major" orientation and 10% of the protein binds heme in the "minor" orientation (pH 7.0, 25 °C) with $\Delta H° = -2.9 \pm 0.3$ kcal/mol and $\Delta S° = -5.4 \pm 0.9$ eu for this equilibrium.

Each day, 1–3% of the hemoglobin in the mature erythrocyte is converted to methemoglobin (Eder et al., 1949), which cannot function in the transport of dioxygen. The enzyme NADPH–methemoglobin reductase (Scott et al., 1965) catalyzes the reduction of methemoglobin to deoxyhemoglobin and prevents accumulation of an undesirable level of methemoglobin over the 120 day lifespan of the erythrocyte. In 1971, Hultquist and Passon demonstrated the presence of small amounts of a soluble form of cytochrome b_5 in human erythrocytes and established that catalytic amounts of this cytochrome greatly accelerated the reduction of methemoglobin by NADPH–methemoglobin reductase. This observation combined with subsequent immunological (Kuma et al., 1976) and functional (Hultquist et al., 1978) studies established that NADPH–methemoglobin reductase is, in fact, a cytochrome b_5 reductase. Consequently, the reduction of methemoglobin to deoxyhemoglobin is now known to result from the direct reaction of the subunits of methemoglobin with ferrocytochrome b_5 that is produced in turn by "methemoglobin reductase."

The concentration of the cytochrome in the erythrocyte is quite low (Passon et al., 1972), indicating that only catalytic quantities of cytochrome b_5 are required to maintain a minimal level of methemoglobin *in vivo*. As a result, isolation of this protein (Passon et al., 1972; Hultquist et al., 1974; Yubisui et al., 1988) in the amounts required for structural and functional analysis has kept the number of such studies to a minimum (Kuma, 1981). However, with the recent report of a cloned cDNA isolated from human reticulocytes that encodes erythrocyte cytochrome b_5 (Giordano & Steggles, 1991), the sequence of this cytochrome is now known with certainty, and the production of larger amounts of this protein by recombinant methods is possible. In the present study, we report the preparation of a gene coding for human erythrocyte cytochrome b_5 (HEb_5)[1] which permits the routine production of this protein in quantities that were previously not possible. The remainder of this report provides the first extensive characterization of the structural, functional, and spectroscopic properties of this protein.

EXPERIMENTAL PROCEDURES

Construction of the Human Erythrocyte Cytochrome b_5 Gene. The gene encoding the human erythrocyte form of cytochrome b_5 was prepared by directed mutagenesis of an expression vector encoding lipase-solubilized bovine liver microsomal cytochrome b_5 that was reported previously (Funk et al., 1990). Substitutions were accomplished consecutively using a two-step PCR mutagenesis procedure (Nelson & Long, 1989). After each round of substitution, the DNA product was cloned into pUC19 (Messing, 1983), and the DNA sequence of individual clones was determined with the dideoxy double-strand method (Gatermann et al., 1988). Using the numbering system of the lipase-solubilized fragment of bovine liver cytochrome b_5, the following single-residue substitutions were assembled: N17H, Y27H, and L70M. In addition, the amino- and carboxyl-terminal insertions were created to produce the following terminal sequences: N-terminus, MAEQSDEAV; C-terminus, DDRPKLNKPPEP## (Figure 1). The resulting gene cassette was then cloned into pUC19 as described previously (Funk et al., 1990) which was then used to transform the *Escherichia coli* host strain JM83. However, the level of recombinant protein expression from

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[1] Abbreviations: HEb_5, human erythrocyte cytochrome b_5; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TAPS, *N*-[[tris(hydroxymethyl)methyl]-3-amino]propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride.

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this vector was minimal. Subsequently, a partially degenerate oligonucleotide(5'-AGGAGAACA[A/C][C/A/T][C/A/T]-ATGGCNGA[A/G]AG[C/T]GA[C/T]GA[C/T]-GAAGCTGTC-3') was designed to allow for the partial randomization of the DNA sequence between the ribosome binding site and the sixth condon of the protein coding sequence. A subsequent PCR reaction using this oligonucleotide and the m13 forward primer (which lies distal to the 3' end of the cytochrome b_5 gene cassette) resulted in a mixed-population PCR product which was then cloned as before into the pUC19 expression vector. Following transformation of *E. coli* JM83, individual clones were grown overnight, and a single red-colored clone was selected for DNA sequence analysis. A single-nucleotide substitution was observed in the sequence of this clone that resulted in changing the codon for Ala2 from GCC to GCA.

Protein Expression and Purification. A single colony of *E. coli*, JM101, containing the recombinant plasmid was grown for 4 h at 37 °C in 2 mL of superbroth medium containing glycerol (1 mL/L) and ampicillin (100 mg/L). This culture (0.5 mL) was used to inoculate 100 mL of the same medium, and the cells were grown for 4 h under the same conditions. Each of 10 2 L Erlenmeyer flasks, containing 1.6 L of superbroth medium plus glycerol (1 mL/L) and ampicillin (100 mg/L), was inoculated with 5 mL of the previous culture, and the cells were grown at 37 °C for 24 h with vigorous shaking. After this time, the shaking speed was reduced to decrease the level of aeration to a minimal level (Funk et al., 1990), and the cultures were incubated for a further 12 h. The cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris, 1 mM EDTA, and 1 mM PMSF, pH 7.5), lysozyme (1 mg/mL) was added, and the mixture was incubated with shaking for 1 h at 4 °C. The suspension was frozen and thawed overnight at 4 °C, $MgCl_2$ (50 mM), deoxyribonuclease (Sigma, D-5025) (0.05 mg/mL), and ribonuclease (Sigma, R-4875) (0.02 mg/mL) were added, and the mixture was incubated at 4 °C for 1 h, with gentle shaking. The cell debris was removed by centrifugation at 9000*g* for 30 min (4 °C), and the supernatant fluid was brought to 50% $(NH_4)_2SO_4$ saturation and stirred for 1 h. The suspension was centrifuged at 9000*g* for 30 min, and the supernatant fluid was dialyzed exhaustively against water. The dialysate was clarified by centrifugation at 9000*g*, loaded onto a column (4 × 5 cm) of DE-52 cellulose (Whatman) (4 °C) equilibrated with 20 mM sodium phosphate buffer (pH 7.2), washed with 5 column volumes of the same buffer, and eluted with 150 mM sodium phosphate buffer (pH 7.2). Fractions containing cytochrome b_5 were concentrated and exchanged into 20 mM sodium phosphate buffer (pH 7.2) by centrifugal ultrafiltration, and loaded onto a column (2.5 × 100 cm) of Sephadex G-75 fine (4 °C) that was equilibrated and then developed with the same buffer. Final purification of the cytochrome was achieved by elution over an HR 10/10 Mono-Q FPLC column (Pharmacia) (ambient temperature) in 20 mM triethanolamine (pH 7.3) with a linear gradient of 0.17–0.22 M NaCl. The yield of purified cytochrome ($A_{413}/A_{280} \geq 6.4$) was ~20 mg/L of culture.

Electrospray Mass Spectrometry. Electrospray mass spectrometry was performed with a triple quadrupole mass spectrometer (API III MS/MS system; Sciex, Thornhill, Ontario, Canada) fitted with a pneumatically assisted electrospray interface (Feng & Konishi, 1992). Samples (1 mL) were prepared in water to a concentration of ~1 mg/mL.

Electronic Absorption Spectroscopy. Electronic spectra were acquired with a Cary 219 spectrophotometer interfaced to a microcomputer (OLIS, Bogart, GA) and fitted with a circulating thermostated water bath. Absorption coefficients were determined as described previously (Antonini & Brunori, 1971).

EPR Spectroscopy. EPR spectra were obtained at X-band frequencies with a Bruker ESP 300 E spectrometer equipped with an Oxford Instruments Model 900 continuous flow liquid helium cryostat, an Oxford Instruments ITC4 temperature controller, and a Hewlett-Packard Model 5352B frequency counter; experimental conditions: 10 K, 0.99 mW microwave power, 9.45 GHz microwave frequency, 100 kHz modulation frequency, and 0.51 mT modulation amplitude.

Potentiometric Titrations. Potentiometric titrations were performed as described previously (Barker et al., 1991) with cytochrome b_5 samples (0.2 and 0.35 μmol) that had been exchanged into 0.10 M NaCl by centrifugal ultrafiltration (Centriprep 10, Amicon). Titrants were CO_2-free 0.01 M HCl in 0.09 M NaCl or 0.01 M NaOH in 0.09 M NaCl. Stock solutions of base were standardized against potassium acid phthalate (primary standard, Aldrich) and used to standardize stock solutions of acid. The proteins were titrated at 25 °C from pH 5.6 to 10.0 and then titrated from pH 10.0 to 5.0.

Electrochemistry. Reduction potentials were measured by direct electrochemistry (Bagby et al., 1990) at a gold electrode modified with the peptide KCTCCA (Sigma, L-4512) (Barker et al., 1990). Samples of protein (~300 μM) for variable temperature experiments were exchanged into sodium phosphate buffer (μ = 0.10 M, pH 7.0). For variable pH experiments (25.0 °C), a mixed-buffer system consisting of MES (5 mM), MOPS (5 mM), TAPS (5 mM), and NaCl (95 mM) was used (Barker & Mauk, 1992). Reduction potentials were converted to the hydrogen scale as described by Dutton (1978). The uncertainty of midpoint potentials determined by direct electrochemistry is estimated to be ±2 mV.

^1H NMR Spectroscopy. ^1H NMR spectra were recorded at 20 °C with a Bruker MSL-200 spectrometer operating in the quadrature mode at 200 MHz. A total of 3000 transients were collected over a 38.5 kHz bandwidth with 4096 data points, using a superWEFT (Inubushi & Becker, 1983) with a recycle delay of 120 ms. Before Fourier transformation, the free induction decay was zero-filled to 8192 data points and multiplied by an exponential function which introduced 0–5 Hz line broadening. For pH titration experiments, samples were prepared in 0.10 M NaCl and adjusted to the desired pH* (uncorrected pH-meter reading) as measured with a Radiometer Model 84 pH meter and an Aldrich (Z-11 343–3) microcombination electrode by addition of 0.1 M D_3PO_4 or NaOD. For analysis of the heme orientational disorder, cytochrome samples were prepared in 50 mM sodium phosphate, pH* 7.0, and then equilibrated at the desired temperature until equilibration was achieved.

RESULTS

Gene Preparation and Protein Expression. The gene encoding for HEb_5 was constructed by directed mutagenesis of the synthetic gene of the lipase-solubilized bovine liver microsomal cytochrome b_5 (Funk et al., 1990). The nucleotide sequences of the two genes as well as the corresponding amino acid sequences are shown in Figure 1. Apart from the differences in the N-terminus, which include the presence of four extra residues in HEb_5, this protein contains eight amino acid substitutions relative to the sequence of lipase-solubilized bovine b_5. These differences include the incorporation of two additional histidine residues, at positions 17 and 27, which

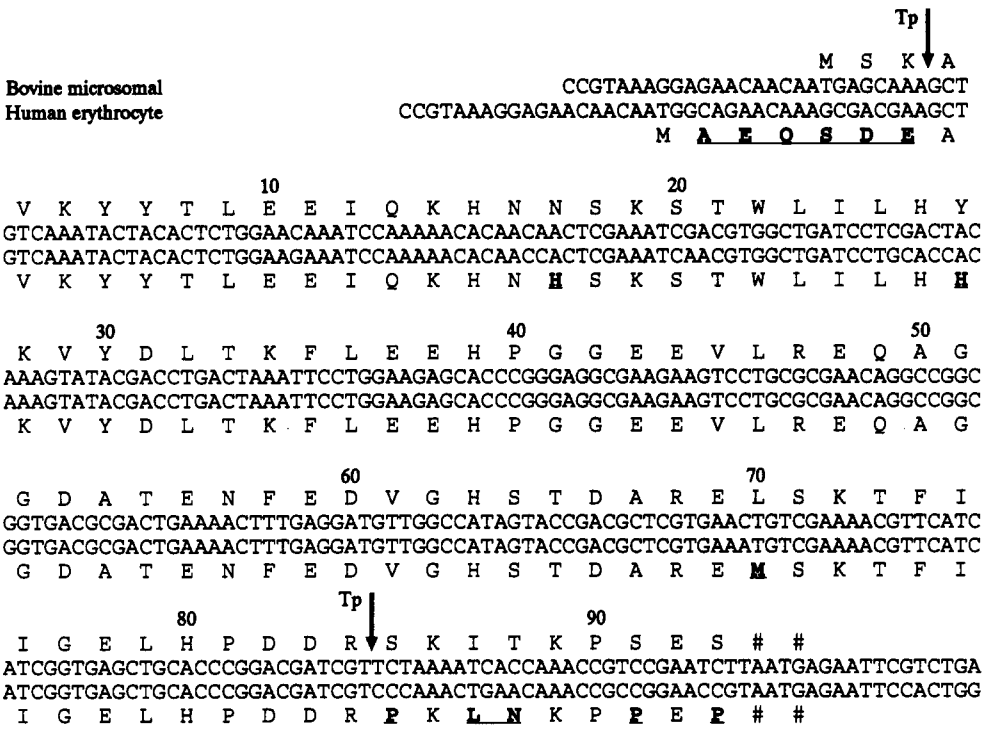

FIGURE 1: Nucleotide sequences of the synthetic genes encoding for the lipase-solubilized fragment of microsomal bovine cytochrome b_5 (Funk et al., 1990) and HEb_5 and the corresponding amino acid sequences of the two proteins. The residue numbering is that of Mathews (1985). The differences between the two protein sequences are highlighted. The sites of cleavage by trypsin in the bovine protein are denoted by Tp.

should be located on the surface of the protein in the absence of any substitution-induced major conformational change. The level of expression in *E. coli* of the recombinant human cytochrome using the vector originally constructed was minimal. However, conversion of the codon for Ala-2 from GCC to GCA resulted in much higher levels of expression. We were subsequently able to obtain yields of ~20 mg of purified protein per liter of culture.

Mass Spectrometry. Electrospray mass spectrometry of purified HEb_5 indicated that the molecular mass of the protein is 11 134 ± 2 daltons. This value corresponds to the molecular mass expected for the apocytochrome without the N-terminal methionine residue (11 137 daltons). Purified HEb_5 was found to contain just a single component, with no evidence for partial cleavage of the N-terminal methionine.

Electronic Absorption Spectroscopy. The electronic spectrum of HEb_5 is typical of that for *b*-type cytochromes. The ferricytochrome exhibits absorption maxima at 413 (ϵ = 122 mM^{-1} cm^{-1}), 532 (ϵ = 10.1 mM^{-1} cm^{-1}), and 560.5 (ϵ = 8.4 mM^{-1} cm^{-1}) nm, and the ferrocytochrome maxima occur at 423.5 (ϵ = 185 mM^{-1} cm^{-1}), 527 (ϵ = 14.1 mM^{-1} cm^{-1}), and 555.5 (ϵ = 26.4 mM^{-1} cm^{-1}) nm [20 mM sodium phosphate buffer (pH 7.2), 25.0 °C]. These adsorption maxima are virtually identical to those reported by Passon et al. (1972) (ferricytochrome, 413 nm; ferrocytochrome, 423, 527, and 556 nm) for samples of this protein isolated from human erythrocytes. Purified HEb_5 eluting from FPLC anion-exchange columns exhibited $A_{413}/A_{280} \geq 6.4$ and exhibited a single band by SDS–polyacrylamide gel electrophoresis. Ratios as high as 6.57 reported by Yubisui and co-workers (Yubisui et al., 1988) were not observed.

EPR Spectroscopy. The EPR spectrum of oxidized HEb_5 at 10 K exhibits *g* values of 3.06, 2.22, and 1.42 that are characteristic of a low-spin ferric heme iron with bishistidine coordination. These values are very similar to those obtained for samples isolated from natural sources of human erythrocytes (*g* values: 3.03, 2.21, 1.39; Passon et al., 1972), recombinant rat microsomal cytochrome b_5 (3.05, 2.24, and 1.46; Beck von Bodman et al., 1986), and recombinant rat outer mitochondrial membrane cytochrome b_5 (3.03, 2.24, and 1.46; Rivera et al., 1992). We conclude, therefore, that HEb_5 has a bishistidine heme coordination environment that is essentially identical to that found in other members of the cytochrome b_5 family (Bois-Poltoratsky & Ehrenberg, 1967).

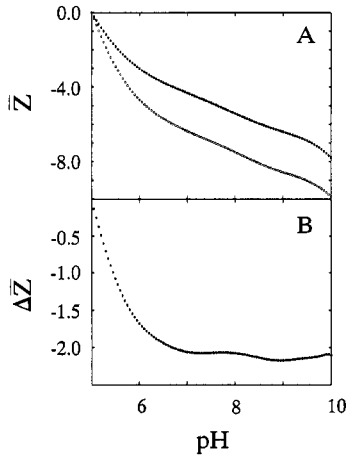

FIGURE 2: Potentiometric titration curves of cytochrome b_5 in 0.10 M NaCl at 25.0 °C. $\bar{Z}$ is the net proton charge (Tanford, 1962); the pH of zero net charge was arbitrarily taken as 5.0 for both proteins. (A) Titration curve of bovine liver microsomal cytochrome b_5 (closed circles) and human erythrocyte cytochrome b_5 (open circles). (B) Difference titration curve generated by subtraction of the titration curve of the bovine cytochrome from that of the human erythrocyte curve.

Potentiometric pH Titration. The proton titration curves for bovine liver microsomal tryptic and human erythrocyte ferricytochromes b_5 are shown in Figure 2. The titration of the protein is fully reversible between pH 5.6 and 10 with no evidence of hysteresis. The difference titration curve obtained by subtraction of the trypsin-solubilized bovine cytochrome b_5 from that of the human erythrocyte cytochrome indicates the presence of two additional ionizable groups in HEb_5 that titrate between pH 5 and pH 7. These groups are presumed

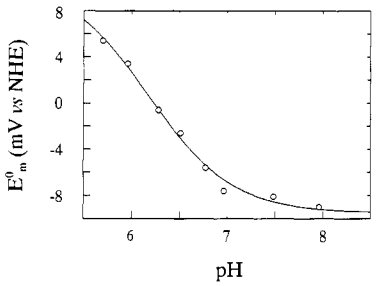

FIGURE 3: Dependence of the midpoint reduction potential of HEb_5 on pH at 25.0 °C. The solid line is the theoretical fit of the data to eq 1.

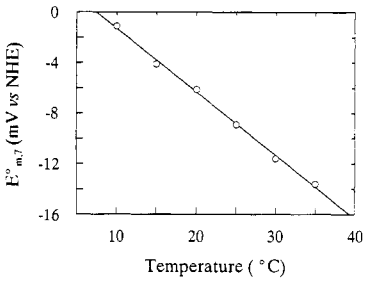

FIGURE 4: Temperature dependence of the midpoint reduction potential of HEb_5 at pH 7.0 (phosphate), μ = 0.10 M.

to be histidines-17 and -27 that are present in HEb_5 but not in bovine liver microsomal tryptic b_5.

Electrochemistry. At pH 7.0 (sodium phosphate buffer), μ = 0.10 M, and 25.0 °C, the reduction potential of HEb_5 determined by cyclic voltammetry is −9 ± 2 mV (vs SHE). The variation of the reduction potential of HEb_5 with pH is shown in Figure 3. These data can be fitted to eq 1 (Reid et al., 1982), which assumes the existence of a heme-linked ionizable functional group that undergoes a change in pK_a when the protein changes oxidization state.

$$E_m^0 = E + \frac{RT}{nF}\ln\left(\frac{K_{red} + [H^+]}{K_{ox} + [H^+]}\right) \quad (1)$$

In this equation, E_m^0 represents the reduction potential at any given pH, and E represents the reduction potential of the fully protonated form. According to the model, protonation of the titrating group should stabilize the reduced form and result in an increased reduction potential, as seen in Figure 3. The solid line shown in Figure 3 represents a nonlinear least-squares fit of the data to eq 1 and gives values for pK_{ox} and pK_{red} of 6.0 ± 0.1 and 6.3 ± 0.1, respectively, with E = 11 ± 2 mV. Previous work on trypsin-solubilized bovine liver cytochrome b_5 gives values for pK_{ox} and pK_{red} of 5.8 and 6.2, respectively (Reid et al., 1982).

The temperature dependence of the reduction potential at pH 7.0, μ = 0.10 M is shown in Figure 4. The thermodynamic parameters derived for this equilibrium from these data were determined (Taniguchi et al., 1980) to be $\Delta H°$ = −3.2 ± 0.1 kcal/mol and $\Delta S°$ = −11.5 ± 0.3 eu. The corresponding parameters for trypsin-solubilized bovine liver microsomal cytochrome b_5 were found to be (Reid et al., 1982) $\Delta H°$ = −11 ± 1 kcal/mol and $\Delta S°$ = −37 ± 2 eu (pH 7.0, μ = 0.10 M).

^{1}H NMR Spectroscopy. The 200 MHz ^{1}H NMR spectrum of HEb_5 is shown in Figure 5. This spectrum is similar to those previously published for the trypsin-solubilized fragment of bovine liver microsomal cytochrome b_5 (McLachlan et al., 1986a, 1988) and is indicative of a low-spin ferric heme iron center as expected for this protein.

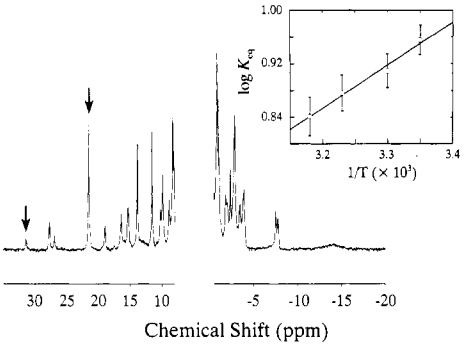

FIGURE 5: 200 MHz ^{1}H NMR spectrum of HEb_5 at 20 °C and pH* 6.99. The 3′-Me (31.5 ppm) protons of the "minor" isomer (see text) and the 5-Me (21.6 ppm) protons of the "major" isomer are indicated by arrows.

Previous studies have established that following reconstitution of apocytochrome b_5 with hemin an equilibrium is established between two forms of the protein that differ in the orientation of the heme group by a 180° rotation about the α,γ-meso axis (Keller & Wüthrich, 1980; La Mar et al., 1981; McLachlan et al., 1986a). The presence of significant amounts of the so-called "minor" isomer in HEb_5 is apparent from the large number of peaks present in the low-field portion of the spectrum and by comparison with previously published studies of the trypsin-solubilized bovine liver cytochrome (McLachlan et al., 1986a, 1988). Although we have not assigned the paramagnetically shifted heme resonances of HEb_5, comparison with previously published assignments (McLachlan et al., 1986a, 1988) permits tentative assignment of the resonances at 31.5 and 21.6 ppm (indicated by arrows in Figure 5) to the 3′-Me of the "minor" orientation and the 5-Me of the "major" orientation, respectively. Integration of these peaks provides the equilibrium constant, K_{eq}, for the interconversion of these two conformers. For HEb_5, K_{eq} = 9.0 ± 0.1 at 25.0 °C. The thermodynamic parameters for this equilibrium obtained from the variation of K_{eq} with temperature (Figure 5, inset) are $\Delta H°$ = −2.9 ± 0.3 kcal/mol and $\Delta S°$ = −5.4 ± 0.9 eu (assuming an error of ±0.1 in the values of log K_{eq}). The small deviation from complete linearity in the plot of log K_{eq} vs $1/T$ may indicate that $\Delta H°$ is temperature-dependent. From these values for $\Delta H°$ and $\Delta S°$, a value of ΔG_{298} = −1.3 ± 0.4 kcal/mol is obtained. The corresponding thermodynamic parameters determined in control experiments with the trypsin-solubilized fragment of bovine liver microsomal cytochrome b_5 were $\Delta H°$ = −1.4 ± 0.3 kcal/mol, $\Delta S°$ = −0.3 ± 0.7 eu, and ΔG_{298} = −1.3 ± 0.2 kcal/mol (Lee et al., 1990).

The variation of the ^{1}H NMR spectrum of human erythrocyte cytochrome b_5 between pH 5.4 and 9.1 is shown in Figure 6. The chemical shifts of most of the heme resonances are essentially independent of pH, with the exception of the two 6-α protons of the 6-propionate group and the 5-Me protons. This type of behavior has been observed previously for trypsin-solubilized bovine liver cytochrome b_5 (McLachlan et al., 1986b). These latter results can be fitted to the Henderson–Hasselbach equation (assuming a single ionizing group) to give a pK_a for the titrating group of 5.5 ± 0.2, which compares with the corresponding value obtained for the bovine protein of ~5.9 (McLachlan et al., 1986b).

DISCUSSION

The present report describes the preparation and expression in *E. coli* of a gene encoding human erythrocyte cytochrome b_5. We are able to obtain yields of this protein in significantly greater amounts than was possible using previously published

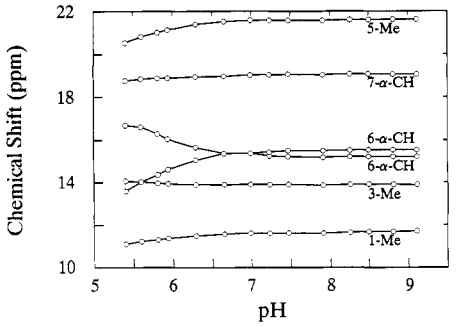

FIGURE 6: pH dependence of the hyperfine-shifted resonances of the major component of HEb_5 (^2H_2O, 20 °C). Tentative assignments of the resonances are indicated.

procedures (Passon et al., 1972; Hultquist et al., 1974; Yubisui et al., 1988), and this has allowed us to examine in greater detail the structural and functional subtleties of this molecule. As indicated above, the recombinant human erythrocytic cytochrome was undetectable by SDS–PAGE analysis following attempted expression from bacteria transformed with the initial gene construction produced by simple conversion of the bovine liver cytochrome expression plasmid. Studies of the expression of SV40 t antigen from plasmids have indicated that protein expression levels can be affected dramatically by changes in the sequences at or near the ribosome binding site (RBS) or the initiator methionine (Iserentent & Fiers, 1980; Gheysen et al., 1982). These effects on expression can vary over several orders of magnitude and may arise from inhibitory RNA secondary conformations that can presumably inhibit translation by the ribosome. These considerations led us to perform a randomized mutagenesis study in which neutral or silent substitutions were made that span the region between the RBS and the first codons of the message. Upon transformation, a single red-colored clone was analyzed by DNA sequencing and was found to have a substituted codon for Ala-2 (GCC-GCA). A survey of highly expressing clones would be required to determine whether a substitution pattern could be discerned. Nevertheless, the current results confirm that nucleotide sequences within this region can have dramatic effects on protein expression levels.

In the absence of crystallographic three-dimensional characterization of the recombinant human cytochrome, potentiometric titrations provide a sensitive and efficient means of assessing (Barker et al., 1991) the electrostatic differences between the recombinant human erythrocyte cytochrome and the more extensively characterized bovine liver cytochrome. Comparison of the potentiometric titrations of the erythrocyte and bovine liver microsomal tryptic cytochrome b_5 (Figure 2) reveals the presence of two additional groups titrating between pH 5 and 7 in the erythrocyte cytochrome, which is consistent with the presence of two additional histidine residues (His-17 and -27) in this protein. The origin of the small difference centered at pH 8 is not clear. Only two ionizable groups in cytochrome b_5 have been suggested to titrate between pH 6.5 and 8.5: His-26 and the α-amino group. For bovine cytochrome b_5, the pK_a of His-26 has been calculated to be 7.5 at low ionic strength (μ = 4 mM, 25 °C; Mauk et al., 1986), while NMR measurements have indicated a value of 6.9 at higher ionic strength (μ = 75 mM, 24 °C; Altman et al., 1989). No direct measure of the α-amino pK_a has been reported, but electrostatic calculations suggest a value of 8 (μ = 4 mM, 25.0 °C; Mauk et al., 1986). The presence of His-27 in the human cytochrome, adjacent to His-26, could perturb the electrostatic environment of His-26 sufficiently to shift the pK_a of this residue to a detectably different value. However, from the information available currently concerning the normal pK_a of His-26, such a shift would have to be ~1–1.5 pK_a units to produce the small perturbation seen around pH 8 in the difference titration curve reported in Figure 2B. At present, there is no reason to suspect that the pK_a of the α-amino group should be different in the bovine liver and human erythrocyte cytochromes. While we cannot provide a definitive explanation for this observation at this time, the effect is, in any event, small and is not inconsistent with the general conclusion that the structures of the bovine liver and human erythrocyte cytochromes are highly similar to each other over the range of pH studied in these titrations.

The differences in sequence between erythrocyte and lipase-solubilized cytochrome b_5 have only a marginal effect on the reduction potential. The reduction potential of HEb_5 (pH 7.0, μ = 0.10 M) was found to be −9 ± 2 mV compared to a value for lipase of +4 mV (Barker et al., 1993). One previous report (Abe & Sugita, 1979) indicated a midpoint reduction potential for the human erythrocyte cytochrome of −2 mV which did not vary between pH 6 and 8. In the present work, however, we find that the midpoint potential of this protein does exhibit a small variation with pH that is consistent with the presence of an ionizable group that undergoes an oxidation-state linked change in pK_a. This discrepancy between these two studies may arise from the inability of the ferri/ferrocyanide technique used in the previous report to detect small changes in potential of an equilibrium with such a low reduction potential. Similar pH-dependent electrochemical behavior has been observed previously for trypsin-solubilized bovine liver microsomal b_5 (Reid et al., 1982) and attributed to the presence of a heme 7-propionate group which is oriented toward the iron in the oxidized state (Argos & Mathews, 1975). Subsequent electrochemical characterization and dimethyl ester protoheme IX-substituted cytochrome b_5 established that this explanation was incorrect (Reid et al., 1984). Alternatively, this pH-dependent behavior may result from the redox-linked titration of a His residue, but as the pK_a values of these groups have not been determined for the ferrocytochrome, this possibility cannot be assessed adequately at present. Whatever the residue responsible for this behavior may be, the pK_a values of this group are increased slightly in the case of the erythrocyte protein.

The proton NMR spectrum of human erythrocyte b_5 is similar to that previously reported for the trypsin-solubilized fragment of bovine liver cytochrome b_5 (McLachlan et al., 1986a, 1988). As reported previously for other b-type cytochromes (La Mar et al., 1981; McLachlan et al., 1986a, 1988; Lee et al., 1990; Rivera et al., 1992), the large number of peaks in the low-field portion of the spectrum indicates the presence of a significant degree of heme rotational disorder. The ratio of the two conformers observed for HEb_5 was 9.0 ± 0.1, which is nearly identical to the value of 8.9 reported for the trypsin-solubilized fragment of bovine liver ferricytochrome b_5 (McLachlan et al., 1986a). Corresponding equilibrium ratios reported for other species of cytochromes b_5 are 10 for rabbit, 20 for chicken, and 1.6 for recombinant rat (La Mar et al., 1981; Lee et al., 1990). It has been suggested (Rivera et al., 1992) that the greater stability of the "minor" form in recombinant rat cytochrome b_5 may result from the manner in which the protein is folded in the *E. coli* host, resulting in conformational arrangements that selectively stabilize the reverse form. As the ratio we obtain from the present recombinant expression system does not appear to be similarly anomalous and as we obtain similar results with both tryptic and lipase-solubilized recombinant bovine liver cytochrome b_5 (Ferrer and Mauk, unpublished results), we have no reason to suspect that bacterial expression of these

proteins under the conditions employed in this work has any influence on the heme orientation equilibrium. However, the lack of corresponding NMR studies of nonrecombinant human erythrocyte cytochrome b_5 prevents direct comparison, and we are therefore unable to confirm this conclusion. The enthalpic and entropic contributions to this equilibrium dictate that the minor isomer is favored as temperature increases. Similar behavior has been observed for the trypsin-solubilized fragment of bovine liver microsomal cytochrome b_5 (Lee et al., 1990).

The ^{1}H NMR pH titration behavior of HEb_5 is essentially the same as that of the trypsin-solubilized bovine protein (McLachlan et al., 1986b) despite the presence of two additional His residues and four additional residues at the carboxyl terminus of the erythrocyte protein. The titratable group responsible for this behavior has been assigned (McLachlan et al., 1986b) as the 6-propionate group of the heme. The pK_a value for the erythrocyte protein was found to be slightly lowered (pK_a = 5.5 ± 0.2) compared to the value for the trypsin-solubilized fragment [pK_a ~ 5.9 (McLachlan et al., 1986b)], in contrast to the relative magnitudes of the pK_as for the 7-propionate (see above). It appears, therefore, that the exact orientation and environment of each propionate group are altered slightly when compared with the trypsin-solubilized bovine protein and that this is reflected in differences in their pH titration behavior.

The development of an efficient expression system for production of human erythrocyte cytochrome b_5 provides the first convenient means of obtaining this protein in the amounts required for functional and structural studies. With the definition of the fundamental characteristics of this protein in the current study, we anticipate the subsequent use of this protein in the detailed characterization of the interaction of this cytochrome with its physiological redox partner, human methemoglobin. Studies directed toward this objective are currently in progress.

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