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Pressure-Induced Conformational Changes in a Human Bence–Jones Protein (Mcg)†

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ABSTRACT: The effect of high static pressures on the internal structure of the immunoglobulin light chain (Bence–Jones) dimer from the patient Mcg was assessed with measurements of intrinsic protein fluorescence polarization and intensity. Depolarization of intrinsic fluorescence was observed at relatively low pressures (<2 kbar), with a standard volume change of −93 mL/mol. The significant conformational changes indicated by these observations were not attributable to major protein unfolding, since pressures exceeding 2 kbar were required to alter intrinsic fluorescence emission maxima and yields. Fluorescence intensity and polarization measurements were used to investigate pressure effects on the binding of bis(8-anilinonaphthalene-1-sulfonate) (bis-ANS), rhodamine 123, and bis(*N*-methylacridinium nitrate) (lucigenin). Below 1.5 kbar the Mcg dimer exhibited a small decrease in affinity for bis-ANS (standard volume change ~5.9 mL/mol). At 3 kbar the binding activity increased by >250-fold (volume change −144 mL/mol) and remained 10-fold higher than its starting value after decompression. With rhodamine 123 the binding activity showed an initial linear increase but plateaued at pressures >1.5 kbar (standard volume change −23 mL/mol). These pressure effects were completely reversible. Binding activity with lucigenin increased slightly at low pressures (standard volume change −5.5 mL/mol), but the protein was partially denatured at pressures >2 kbar. Taken in concert with the results of parallel binding studies in crystals of the Mcg dimer, these observations support the concept of a large malleable binding region with broad specificity for aromatic compounds. The complementarity between this binding region and a suitable ligand can be improved by the imposition of external pressures far below those causing general disruption of the protein structure.

The light chain found in the Bence–Jones dimer and monoclonal IgG1 protein from the patient Mcg exhibits a high degree of conformational flexibility. Three-dimensional structural studies revealed that the Mcg dimer contained two light chains identical in amino acid sequence but folded in different conformations (Schiffer et al., 1973; Edmundson et al., 1975). Noncovalent interactions between the two variable domains produced a hydrophobic binding cavity. The Bence–Jones dimer formed trigonal crystals in ammonium sulfate and orthorhombic crystals in water (Abola et al., 1980; Ely et al., 1983). Conformational differences were found in the crystal structures of analogous light chains in the trigonal and orthorhombic forms, an indication that at least four different conformational isomers existed in the light chain dimers. Another conformational isomer was observed in the X-ray analysis of the Mcg IgG1 protein (Rajan et al., 1983).

In this report we consider the question of analogous conformational flexibility of the Mcg light chain in aqueous solution. Environmental perturbation of proteins in solution can be investigated by varying either temperature or pressure. Temperature studies produce changes in both protein thermal energy and protein volume, and it is difficult to separate the

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two effects (Weber & Drickamer, 1983). Additionally, the effective temperature range is limited by protein denaturation. Pressure studies permit consideration of volumes and thereby intramolecular distances in a controlled, isothermal environment (Weber & Drickamer, 1983). Recent advances in fluorescence technology have enabled the measurement of both fluorescence intensity and polarization over a pressure range of 12 kbar. This technology has been invaluable in the investigation of pressure-induced conformational changes in aqueous protein solutions (Li et al., 1976a,b; Visser et al., 1977; Torgerson et al., 1979; Chryssomallis et al., 1981; Paladini & Weber, 1981a). In the case of the Mcg dimer, conformational flexibility was assessed by following its affinity for three different fluorescent ligands over a pressure range of 3 kbar.

Experimental Procedures

Preparation of Bence–Jones Dimers. Urine samples from a patient (Mcg) with amyloidosis were kindly provided by Dr. Harold F. Deutsch of the University of Wisconsin. The λ-type Bence–Jones protein was isolated and purified by procedures described previously (Edmundson et al., 1984).

Fluorescent Ligands. Rhodamine 123, bis(*N*-methylacridinium nitrate) (lucigenin), and 8-anilinonaphthalene-1-sulfonate (ANS)[1] were obtained from Molecular Probes (Junction, OR) and used without further purification. Purified samples of bis(8-anilinonaphthalene-1-sulfonate) (bis-ANS) were kindly provided by Dr. Gregorio Weber and Fay Farris of the University of Illinois, Urbana.

Fluorescence Measurements. Technical fluorescence emission spectra and fluorescence intensities were recorded with a ratiometric spectrofluorometer equipped with digital bipolar averaging circuitry (Wehrly et al., 1979). Fluorescence polarization was measured with the photon counting fluorescence polarization instrument described by Paladini & Weber (1981b). This instrument was also used for photon counting fluorescence intensity measurements, when simultaneous polarization and intensity values were required. Spectra were taken under the following conditions: rhodamine 123 was excited at 470 nm and emission recorded in 2-nm increments between 480 and 650 nm; lucigenin was excited at 430 nm and emission recorded between 450 and 650 nm; ANS and bis-ANS were excited at 370 nm and emission recorded between 400 and 650 nm.

In binding studies, solutions of lucigenin (2 μM), rhodamine 123 (2 μM), and ANS (20 μM) were titrated with the Mcg dimer, the concentration of which was varied over a range of 5–54 μM. Emission spectra were recorded after each addition of protein. Experiments with these ligands were repeated in the following three buffers to ascertain the effects of pH and ionic strength on ligand binding activity: (1) 0.1 M Tris-HCl, pH 8; (2) 0.1 M sodium phosphate, pH 6.2; (3) 0.1 M sodium phosphate–2.5 M NaCl, pH 6.2. Fluorescence intensity values were computed from spectrum integrals and used to construct plots of reciprocal fluorescence yield vs. protein concentration. Affinity constants and maximum quenching/enhancement values were determined by least-squares analyses of these data. In another set of experiments, the Mcg Bence–Jones dimer (20 μM) was titrated with rhodamine 123 (1–38 μM) or lucigenin (1–143 μM). Fluorescence quenching data were analyzed by Scatchard and Hill plots (Scatchard, 1949; Hill, 1910) to assess the valency and cooperativity of the ligand–protein interactions.

High-Pressure Apparatus. Sample solutions were compressed with the high-pressure apparatus described by Paladini & Weber (1981b). Pressure bomb temperature was regulated at 20 ± 1 °C with a Lauda RC3 thermostatic bath. Polarization values were measured with the instrument in the "L" format. At elevated pressures, changes in the birefringency of the pressure bomb windows resulted in apparent depolarization. Birefringency corrections were determined by measuring fluorescence polarization at elevated pressures and −10 °C for a 10^{-6} M solution of fluorescein in concentrated glycerol. Window scrambling factors were computed as suggested by Paladini & Weber (1981b).

A standard protocol was followed in all pressure experiments: pressure was increased in increments of ca. 200 bar through a range of 10^{-3}–3 kbar. Fluorometer readings were allowed to settle for 10–20 min before measurements were recorded. Sample preparations were maintained at maximum pressure for 60 min to test for long-term effects. Pressure was removed in 200–300-bar increments with 10–20-min settling times to assess the reversibility of pressure effects.

Measurement of Intrinsic Protein Fluorescence in the Mcg Bence–Jones Dimer at Elevated Pressures. Emission spectra were recorded by exciting samples of the Mcg dimer (2.5 μM) at 270 nm and scanning the emission monochromator between 290 and 440 nm, in 2-nm increments. For polarization measurements, samples (25.0 μM) were excited at 290 nm through a UV band-pass filter (Corning 7-54) and emission was photon-counted through a 310-nm band-pass filter (Corning 7-60).

Binding of Lucigenin and Bis-ANS to the Mcg Dimer at Elevated Pressures. The effects of high pressure on the binding of these ligands were determined by measuring fluorescence intensity as a function of pressure. The fluorometer configuration was the same as that described above, except for the use of the pressure bomb.

Binding of Rhodamine 123 to the Mcg Dimer at Elevated Pressures. Assessment of rhodamine binding was complicated by an increase in the quenching efficiency of the protein for the ligand at elevated pressures. Calculations of both maximum quenching constant (Q_{max}) and affinity constant (K_a) values at each pressure increment were made possible by simultaneous measurements of fluorescence intensity and polarization with the photon-counting fluorometer [see Herron (1984)]. Rhodamine fluorescence was excited at 470 nm and emission monitored through a 510-nm cutoff filter (Corning 3-69), in lieu of an emission monochromator.

Results

Binding of Fluorescent Ligands to the Mcg Dimer at Atmospheric Pressure. The structural formulas for the fluorescent ligands used in this study are presented in Figure 1. The ligand fluorescence intensity data were used to construct plots of reciprocal fluorescence yield vs. reciprocal protein concentration. Values for affinity constants (K_a), normalized for one ligand binding site per Mcg dimer, are listed in Table I. The values for maximum fluorescence quenching or enhancement factors are also given in Table I. Fluorescence quenching was observed when rhodamine 123 or lucigenin was bound to the Mcg dimer, while enhancement was noted with ANS or bis-ANS.

The affinity of the Mcg dimer for rhodamine 123 was not significantly affected when the pH was changed from 8 to 6.2 (see Table I). However, a 5-fold increase in affinity was observed in 2.5 M NaCl buffered at pH 6.2 with phosphate.

[1] Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonate; bis-ANS; bis(8-anilinonaphthalene-1-sulfonate); V, variable; C, constant; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; 3-D, three dimensional.

Table I: Binding of Fluorescent Ligands to the Mcg Bence–Jones Dimer

binding parameter[a]	buffer: 0.1 M Tris-HCl, pH 8.0	0.1 M phosphate, pH 6.2	0.1 M phosphate–2.5 M NaCl, pH 6.2
		Rhodamine 123	
r^2	0.997	0.997	0.998
affinity	$1.8 \pm 0.1 \times 10^4$	$2.1 \pm 0.1 \times 10^4$	$8.9 \pm 0.2 \times 10^4$
Q_{max} (%)	76 ± 3	54 ± 2	58 ± 1
		Lucigenin	
r^2	0.998	1.000	
affinity	$1.24 \pm 0.05 \times 10^4$	$5.91 \pm 0.04 \times 10^4$	
Q_{max} (%)	82 ± 3	101.4 ± 0.5	
		ANS	
r^2	1.000	0.999	0.998
affinity	1800 ± 130	750 ± 380	5100 ± 900
yield (x-fold)	107 ± 7	130 ± 70	81 ± 14
		Bis-ANS	
r^2	0.999		
affinity	1500 ± 280		
yield (x-fold)	106 ± 19		

[a] Fluorescent ligands were titrated with the Mcg Bence–Jones dimer. Values for affinity constant (M^{-1}) and maximum fluorescence quenching or enhancement (Q_{max} or yield) were obtained by linear regression of plots of reciprocal fluorescence yield vs. reciprocal protein concentration. All affinity values were computed for an univalent Mcg–ligand interaction.

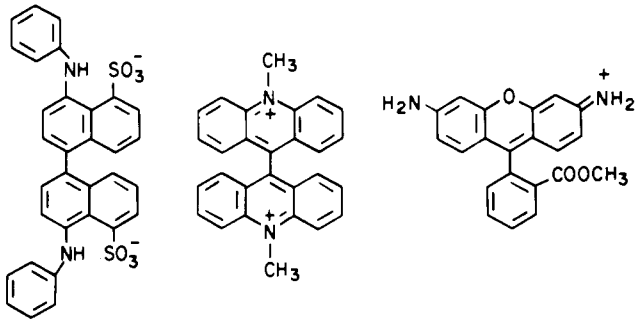

FIGURE 1: Structural formulas of fluorescent ligands. Molecular weights (M_r) are listed for bis-ANS, lucigenin, and rhodamine 123. Molar volumes (V, mL/mol) were computed with Connolly's MS program (1983), which generated molecular surfaces based on van der Waals' molecular radii. With MS, an active site volume of 419 mL/mol was computed for the Mcg Bence–Jones dimer.

The affinity for lucigenin was about 5-fold higher at pH 6.2 than at pH 8. The effect of high ionic strength on the Mcg–lucigenin interaction was not assessed, because lucigenin fluorescence was totally quenched in buffered 2.5 M NaCl. In low salt the affinity for ANS was greater at pH 8 than at 6.2. The highest K_a value was obtained in buffered 2.5 M salt. Values for the K_a and maximum fluorescence enhancement factor for bis-ANS were similar to those for ANS at pH 8 and low salt.

Affinity constants, valencies, and cooperativities determined from Scatchard and Hill plots for rhodamine 123 and lucigenin are listed in Table II. The Mcg dimer exhibited two high-affinity and at least four low-affinity binding sites for rhodamine 123. The high-affinity sites were cooperatively coupled, as evidenced by a cooperativity value of 1.65, and exhibited intrinsic affinity constants of 1.6×10^5 M^{-1} and 5.6×10^6 M^{-1}. Problems with ligand solubility and trivial reabsorption prevented rigorous analysis of low-affinity sites, but available data gave an average intrinsic affinity constant of 4.5×10^4 M^{-1} and a cooperativity index of 0.93.

Similar experiments with lucigenin did not reveal the presence of high-affinity binding sites comparable to those observed for rhodamine 123. However, there were many

Table II: Scatchard and Hill Plot Analyses of the Binding of Rhodamine 123 and Lucigenin to the Mcg Bence–Jones Dimer[a]

binding parameter	rhodamine 123: high-affinity sites	rhodamine 123: low-affinity sites	lucigenin: low-affinity sites
r^2	0.996	0.998	0.998
valence	2	~4	~60
affinity (M^{-1})	1.6×10^5 5.6×10^6	4.5×10^4	730
cooperativity[b]	1.65	0.93	0.99

[a] The Mcg Bence–Jones dimer was titrated with fluorescent ligands, and fluorescence quenching data were analyzed by Scatchard and Hill plots. Rhodamine 123 titrations were performed in 2.5 M NaCl/0.1 M phosphate buffer (pH 6.2), while lucigenin titrations were carried out in 0.1 M phosphate buffer, pH 6.2. [b] Cooperativity values were determined from Hill plots. A value of 1.0 indicates noncooperative binding.

Table III: Pressure Effects on the Intrinsic Fluorescence Intensity and Emission Maxima of the Mcg Bence–Jones Dimer[a]

pressure (kbar)	relative intensity	emission maximum
10^{-3}	1.00	326
0.28	1.01	326
0.55	1.03	327
0.83	1.05	327
1.10	1.06	328
1.38	1.08	328
1.65	1.10	329
1.93	1.13	329
2.21	1.21	330
2.48	1.80	335
2.76	2.06	343
3.03	2.93	346

[a] Relative fluorescence intensity values are normalized to the intrinsic fluorescence intensity at atmospheric pressure. Emission maxima values are reported in nanometers.

(~60) low-affinity binding sites that exhibited an average intrinsic affinity constant of 730 M^{-1} and a cooperativity value of 0.99. On the basis of this analysis all lucigenin binding sites were considered equivalent and independent.

Intrinsic Fluorescence Intensity and Polarization of the Unliganded Mcg Dimer at Elevated Pressures. The measurement of intrinsic fluorescence intensity over a pressure range of 10^{-3}–3 kbar is presented in Table III. Both intrinsic fluorescence intensities and emission maxima were relatively insensitive to pressures lower than 2 kbar. A general im-

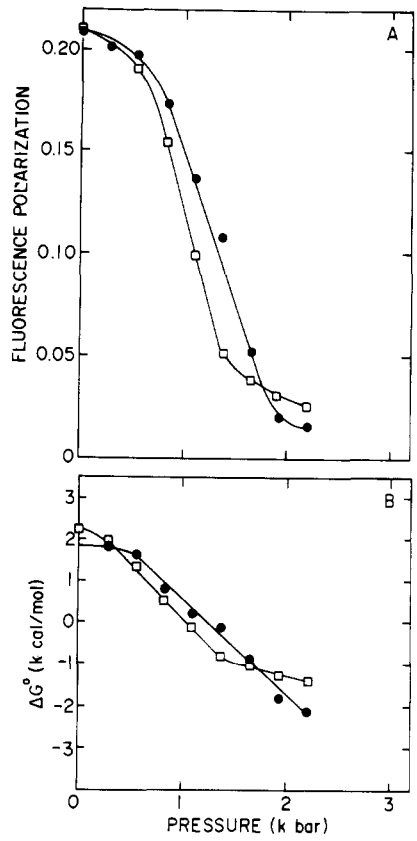

FIGURE 2: Pressure-induced conformational perturbation of the Mcg dimer. (Panel A) The intrinsic fluorescence polarization of the Mcg dimer was observed over a pressure range of 10^{-3}–3 kbar. Samples of unliganded dimer (25.2 μM, in 0.1 M Tris-HCl buffer, pH 8) were excited at 290 nm through a Corning 7-54 UV band-pass filter, and emission was monitored through a Corning 7-60 band-pass filter. Plots are shown for both compression (●) and decompression (□). (Panel B) Standard free energy values ($\Delta G°$) were computed from intrinsic polarization data by using a two-state model discussed in the text. Standard volume changes ($\Delta V°$) computed from the slopes of these plots are listed in Table IV.

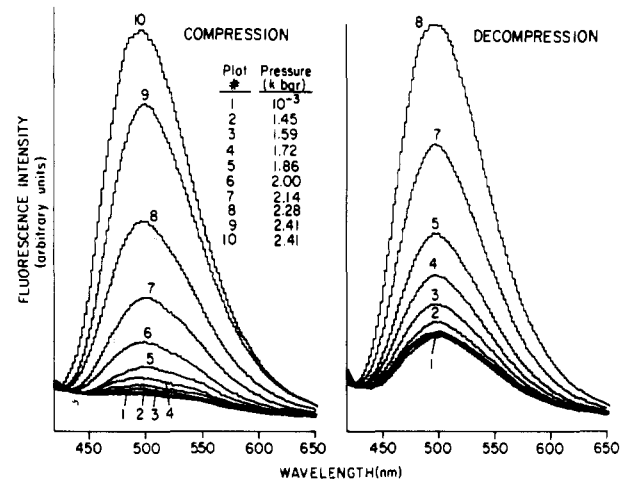

FIGURE 3: Pressure-induced changes in the equilibrium binding of bis-ANS to the Mcg dimer. A solution of bis-ANS (10 μM) and Mcg dimer (18 μM) in 0.1 M Tris-HCl buffer, pH 8, was subjected to pressures of 10^{-3}–3 kbar. Fluorescence emission spectra were recorded for bis-ANS by exciting samples at 370 nm and measuring emission between 420 and 650 nm.

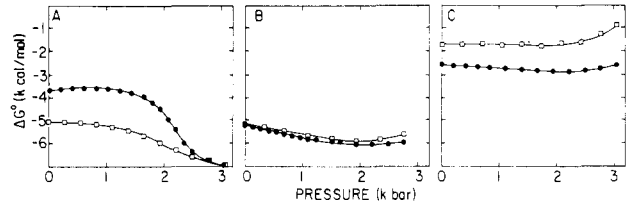

FIGURE 4: Pressure-induced changes in the binding affinity of the Mcg dimer for bis-ANS, rhodamine 123, and lucigenin. Values for $\Delta G°$ were determined with affinity constants calculated from the degree of ligand association (Li et al., 1976a,b). Plots are shown for both compression (●) and decompression (□). Values for $\Delta V°$, computed from the slopes of these plots, are presented in Table IV. (Panel A) Bis-ANS (10 μM) and Mcg dimer (18 μM). (Panel B) Rhodamine 123 (11.1 μM) and Mcg dimer (22.4 μM). Rhodamine 123 fluorescence was excited at 470 nm and emission monitored through a Corning 3-69 cutoff filter. (Panel C) Lucigenin (10 μM) and Mcg dimer (25 μM). Lucigenin fluorescence was excited at 430 nm and emission scanned between 460 and 610 nm. All experiments were performed at 20 °C in 0.1 M Tris-HCl buffer, pH 8.

provement in quantum efficiency and a red shift in emission maxima were observed at higher pressures.

The effects of pressure on the intrinsic fluorescence polarization are shown in Figure 2. A large depolarization was observed at pressures less than 2 kbar. Plots were sigmoidal in shape, suggesting the presence of two stable species: (1) the "native" conformation as found at atmospheric pressure and (2) a "pressure-stabilized" conformation predominating at 2 kbar. Intrinsic polarization data were analyzed in terms of this two-state model for pressure-induced conformational changes. Polarization values were first converted to anisotropy values to permit them to be linearly additive. Anisotropies measured at 1 atm and 2 kbar were used as limiting values. Values for standard free energies ($\Delta G°$) were determined with eq 1 where K_a is the affinity constant calculated from an-

$$\Delta G° = -RT \ln K_a \quad (1)$$

isotropy data (Paladini & Weber, 1981a). Data were plotted as the standard free energy of transition from the native conformation to the pressure-stabilized conformation (see Figure 2).

Standard volume changes ($\Delta V°$) for the conformational transitions were obtained from plots of free energy vs. pressure by using eq 2. For assessment of reversibility, the standard

$$d\Delta G°/dP = \Delta V° \quad (2)$$

volume changes were determined for both compression and decompression plots ($\Delta V°$ values are given in Table IV).

A standard volume change of −93 mL/mol was obtained by linear regression of the compression plot. Comparison of compression and decompression plots showed that pressure-induced conformational effects were only partially reversible (see Figure 2 and Table IV). However, differences in standard free energy before and after pressurization were negligible. The free energy perturbation value between native and pressure-stabilized conformations was −3.8 kcal/mol.

Ligand Binding by the Mcg Dimer at Elevated Pressures. Affinity measurements for bis-ANS, rhodamine 123, and lucigenin were taken over a pressure range of 10^{-3}–3 kbar. Fluorescence data for all three ligands were analyzed by constructing plots of standard free energy ($\Delta G°$) vs. pressure. Values of $\Delta G°$ were determined with affinity constants calculated from the degree of ligand association (Li et al., 1976a,b). Standard volume changes ($\Delta V°$) for the association of ligands with the Mcg Bence–Jones dimer were obtained by least-squares analysis of the slope of each pressure plot (see eq 2).

Both ANS and bis-ANS were initially examined, but bis-ANS was chosen for detailed study because it exhibited more pronounced pressure effects. Moreover, bis-ANS was closer in size to rhodamine 123 and lucigenin. Fluorescence emission

Table IV: Pressure Effects on the Conformation and Ligand Binding Activity of the Mcg Bence–Jones Dimer

	Mcg dimer	bis-ANS	rhodamine 123	lucigenin
volume change[a]				
ΔV°_c	−93 ± 5	5.9 ± 1.1[b]	−23.4 ± 0.6	−5.5 ± 0.4
ΔV°_d	−108 ± 2	−5.8 ± 1.2	−18.2 ± 0.5	0.03 ± 1.2
free energy[c]				
ΔG°_c	1.8 ± 0.2	−3.68 ± 0.01	−5.21 ± 0.01	−2.60 ± 0.01
ΔG°_d	2.2 ± 0.1	−5.00 ± 0.01	−5.17 ± 0.01	−1.71 ± 0.03
ΔG°_m	−2.0 ± 0.1	−6.94 ± 0.01	−6.04 ± 0.01	−2.83 ± 0.02

[a] Standard volume changes (mL/mol) were determined by linear regression of plots of standard free energy vs. pressure (Figures 3 and 4). Values are cited for both compression (ΔV°_c) and decompression (ΔV°_d) plots. [b] Standard volume changes cited for bis-ANS do not reflect the enhanced binding which occurred at higher pressures (see Figure 4). Volume changes of −144 ± 5 and −44 ± 1 mL/mol were computed at 2.5 kbar from compression and decompression plots, respectively. [c] Standard free energy changes (kcal/mol) at atmospheric pressure are listed for compression (ΔG°_c) and decompression (ΔG°_d) plots. Free energy minima (ΔG°_m) were used to calculate the total energy perturbation.

spectra for the binding of bis-ANS are presented in Figure 3. Plots of standard free energy (ΔG°) vs. pressure are shown in Figure 4A.

Bis-ANS binding activity decreased slowly as pressure was increased to 1.5 kbar, giving a ΔV° value of 5.9 mL/mol (see Table IV). However, the affinity increased rapidly in the pressure range 1.5–3 kbar. At 3 kbar, bis-ANS binding was stabilized by more than 3 kcal/mol, relative to atmospheric pressure. A volume change of −144 mL/mol was associated with the enhanced ligand binding. This enhancement was not completely reversed on decompression. After removal of pressure, for example, the binding activity was stabilized by 1.3 kcal/mol, relative to its native value.

Pressure-induced changes in the binding of rhodamine 123 to the Mcg dimer are illustrated in Figure 4B. In contrast to the observation with bis-ANS, a linear decrease in ΔG° was observed at pressures lower than 1.5 kbar. A free energy minimum was observed at 2.2 kbar, where rhodamine 123 binding activity was stabilized by 0.8 kcal/mol, relative to its value at atmospheric pressure (see Table IV). A standard volume change of −23.4 mL/mol was determined for the association of rhodamine 123 to the Mcg dimer. the decompression plot indicated that the observed pressure effects were largely reversible.

Pressure-induced changes in the binding of lucigenin by the Mcg dimer are shown in Figure 4C. The standard volume change was calculated to be −5.5 mL/mol, and the standard free energy was found to be 0.2 kcal/mol lower at 2.2 kbar than at atmospheric pressure (see Table IV). Thus, the binding activity of lucigenin showed a small improvement at elevated pressure. However, an irreversible decrease in affinity occurred after pressure was maintained at 3 kbar for 80 min. Virtually no standard volume change was observed with decompression, and the final ΔG° value was 0.9 kcal/mol higher than its value at atmospheric pressure.

Discussion

Pressure Effects on the Intrinsic Fluorescence Emission Maxima and Intensities of the Mcg Dimer. Tryptophan normally dominates intrinsic protein fluorescence because tyrosine is a weak emitter and its emission is largely quenched by energy transfer to tryptophan (Longworth, 1971). This energy transfer proceeds with an efficiency greater than 50% when the phenol and indole moieties are separated by ≤20 Å (Weber & Teale, 1959; Weber, 1960a). In the crystal structure of the Mcg dimer, each monomer contributes six aromatic side chains to the ligand binding site (four tyrosine and two phenylalanine residues; Edmundson et al., 1974). A single tryptophan residue is buried nearby in the hydrophobic core of each "variable" or V domain. The "constant" or C domain of each light chain contains two tryptophan residues, both inaccessible to solvent in the native structure.

An emission maximum of 348 nm is typically observed for tryptophan in aqueous solution (Weber & Teale, 1957), but this value is shifted to shorter wavelengths when the indole moiety is exposed to environments with lower dielectric constants (Van Duuren, 1961). The pressure-induced shifts in emission maxima (see Table III) indicate that internal fluorophores in the Bence–Jones dimer were not significantly exposed to bulk solvent at pressures less than 2 kbar. The small change (<20%; see Table III) in intrinsic fluorescence intensity at low pressures lends further support to this conclusion. Moreover, solvent exposure was virtually complete by 3 kbar, implying that the dimer was more sensitive to pressure-induced denaturation than chymotrypsinogen, lysozyme, flavodoxin, and riboflavin binding protein (Li et al., 1976a,b; Visser et al., 1977). Denaturation of these proteins required pressures exceeding 6 kbar.

Pressure Effects on Intrinsic Fluorescence Polarization of the Mcg Dimer. Intrinsic fluorescence polarization typically results from local rotation of tryptophan residues during their fluorescence lifetime (Weber, 1960b). The depolarization observed in the Bence–Jones dimer at pressures <2 kbar was probably not attributable to protein denaturation, since depolarization was not accompanied by significant shifts in emission spectra. In earlier studies, Paladini & Weber (1981a) used intrinsic polarization measurements in an assay for subunit dissociation in enolase. They attributed the depolarization after subunit dissociation to increases in the rotational freedom of intrinsic fluorophores. Depolarization may also be partly attributable to increased efficiency in energy transfer between tyrosine and tryptophan residues (Pesce et al., 1971).

The following equation related fluorescence polarization (P) to the intermolecular distance (R) between fluorophores:

$$(1/P - \tfrac{1}{3}) = (1/P_0 - \tfrac{1}{3})[1 + \tfrac{3}{5}(R_0/R)^6] \qquad (3)$$

where P_0 is the intrinsic polarization of the acceptor molecule and R_0 is the critical distance for the donor–acceptor system (e.g., 20 Å for transfer from phenol to indole; Weber & Teale, 1959; Weber, 1960a). Because of the R^6 dependency, small changes in distances between aromatic residues would produce large changes in polarization. In the Mcg dimer, tyrosine residues are particularly important in the interactions across the boundaries of the variable domains (Edmundson et al., 1975). Changes in the intermolecular distances of these residues would affect both domain association and energy-transfer potential.

The intrinsic polarization data for the Bence–Jones dimer reflected conformational changes at relatively low pressures. The standard volume changes observed for the dimer were typical of values reported in the literature for pressure-induced conformational changes and domain dissociation (Li et al.,

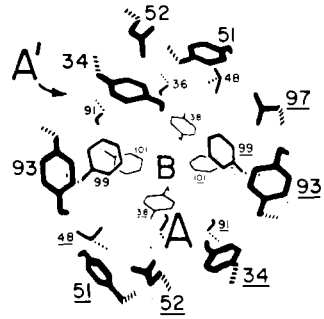

FIGURE 5: Perspective drawing of the side chains lining the main cavity of the Mcg Bence–Jones dimer containing subsites A, A′, and B. Sites A and B are available for binding, but site A′ is blocked by interactions with another dimer in the crystal lattice. The residue numbers of monomer 2 constituents are underlined. The α–β carbon bonds are represented as dotted lines.

1976a; Visser et al., 1977; Paladini & Weber, 1981a). In light of the ligand binding studies discussed below, we conclude that the binding cavity of the Mcg dimer may be rearranged but not destroyed in the pressure-stabilized state.

Binding of Ligands by the Mcg Bence–Jones Dimer at Atmospheric Pressure. A 3-D model of the main binding cavity, as its exists in trigonal crystals (Edmundson et al., 1974), is depicted in Figure 5 to provide a frame of reference for this discussion. Part of the cavity is not available for ligand binding in the crystal lattice because of protein–protein interactions (e.g., subsite A′ in Figure 5). In solution all subsites should be accessible to appropriate ligands.

Two molecules of rhodamine 123 binding cooperatively were probably accommodated in the main cavity, while four or more ligand molecules of lower affinity were located in other parts of the protein (see Table II). The increase in affinity in the presence of 2.5 M NaCl was initially surprising, since rhodamine 123 is positively charged and there are three negatively charged residues on the rim of the main binding cavity of the Mcg dimer. However, in parallel studies in trigonal crystals of the dimer we found that rhodamine 123 would not bind until the crystals were subjected to mild osmotic shock (Edmundson et al., 1984). Moreover, the ligand occupied a shallow position near the entrance of the cavity. In the solution studies we concluded that the masking of the ligand's positive charge in 2.5 M NaCl permitted deep penetration into the active site, where a greater number of aromatic side chains were available for interaction. The simultaneous binding of several ligand molecules in high- and low-affinity sites is not peculiar to the Mcg dimer. For example, bovine serum albumin was found to have four high-affinity and at least eight low-affinity sites for ANS (Daniel & Weber, 1966; Pasby, 1969).

The binding of lucigenin to the Mcg dimer in solution involved multiple low-affinity sites (see Table II). In trigonal crystals the binding was limited to a single site of high occupancy within the main cavity (Edmundson et al., 1984). Specific binding in a crystal was possible because the formal positive charges were neutralized by a sulfate anion which accompanied the ligand into the cavity.

Binding of Bis-ANS, Rhodamine 123, and Lucigenin to the Mcg Dimer at Elevated Pressure. Torgerson et al. (1979) classified binding sites as "hard" and "soft". A hard site cannot decrease its volume and thus exhibits a lower affinity for a ligand as the pressure is increased. A soft site can be compressed, and binding is improved at elevated pressures. Since bond distances and angles are not altered at pressures lower than 12 kbar (Benson & Drickamer, 1957), potential volume changes in proteins occur mainly by rotation about torsion angles. A hard site is typically formed from contiguous regions of polypeptide chain that have very limited torsional flexibility. A soft site is usually produced by polypeptide segments from different subunits or from different parts of the same chain.

At pressures lower than 2 kbar the Mcg dimer possessed a hard site for bis-ANS and soft sites for rhodamine 123 and lucigenin (see Table IV and Figure 4). The standard volume change of 5.9 mL/mol for bis-ANS was similar to the value of 9.3 mL/mol for ANS binding to poly(β-cyclodextrin), a model system for hard sites (Torgerson et al., 1979). Between 2 and 3 kbar of pressure the binding activity for bis-ANS increased 250-fold. The volume change of −144 mL/mol at 2.5 kbar probably reflected large-scale conformational instability of the Mcg dimer. Similar behavior was previously observed in the binding of ANS to chymotrypsinogen and lysozyme (Li et al., 1976a). To explain such observations, Anderson & Weber (1966) proposed that pockets capable of binding charged aromatic molecules were exposed as the proteins unfolded [see also Morero & Weber (1982)].

Pressure-induced association of small aromatic molecules proceeds with a standard volume change of ca. −5 mL/mol (Weber & Drickamer, 1983), a value close to that observed for lucigenin (−5.9 mL/mol; see Table IV). This comparison indicates that lucigenin may be forming molecular complexes with accessible aromatic side chains on the Mcg protein.

The binding of rhodamine 123 to the Mcg dimer proceeds with a much larger decrease in volume (−23.4 mL/mol). This implies some degree of conformational change in the dimer. Even in crystals, where movements of Mcg molecules are dampened by numerous protein–protein interactions, the binding of large ligands like dimers of 6-carboxytetramethylrhodamine is accompanied by expansion of the main cavity and sizable rotations of aromatic side chains to improve the complementarity of protein and ligand (Edmundson et al., 1984).

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Refolding a Disulfide Dimer of Cytochrome *c*†

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ABSTRACT: A covalent dimer of *Saccharomyces cerevisiae* iso-1 cytochrome *c* is stabilized by an interchain disulfide bond involving the cysteine residue penultimate to the C-terminus. The individual chains in the dimer appear to retain the tertiary structural features characteristic for monomeric cytochrome *c* albeit with some perturbation. The dimer is reversibly denatured by heat, urea, or guanidine hydrochloride in a single cooperative transition whose midpoint is less than that of the monomeric protein. The kinetic profile observed for the refolding of the denatured dimer is characteristic for monomeric cytochromes except for a markedly enhanced slow-phase amplitude.

Crystallographic studies of a variety of globular proteins indicate that polypeptide chains having 200 or more amino acid residues are commonly folded into contiguous globular units each containing about 100 residues called domains. It is likely that the individual domains within contemporary proteins once represented independent stable proteins whose structural genes were linked by either gene duplication or gene fusion. In the case of gene duplication, the primary sequences of the linked domains can be quite distinct even though the polypeptide chain fold in each domain remains quite similar. This situation probably reflects the independent genetic drift of the fused structural gene elements, the tolerance of a tertiary fold to sequence variation, and, in some cases, an advantageous asymmetric interaction of adjacent domains.

Cytochrome *c* may be considered to be a protein having a stable single-domain structure (Takano et al., 1977) containing about 100 residues. The domain of all the cytochromes *c* examined can be reversibly unfolded by using denaturants such as urea or guanidine hydrochloride. While most cytochromes *c* do not contain a free sulfhydryl group, the iso-1 isomer purified from *Saccharomyces cerevisiae* contains a single free sulfhydryl group located on a cysteine residue penultimate to the C-terminus (Yaoi, 1967; Narita & Titani, 1969). Formation of a disulfide dimer of the iso-1 monomer would then emulate a two-domain protein resulting from recent duplication of a structural gene. In this report, we inquire whether covalent linkage of two identical polypeptide chains significantly perturbs either the folding or the stability of the individual domains. We use a selectively alkylated monomer to distinguish whether any observed differences between the monomeric and dimeric iso-1 cytochromes result from simple modification of the penultimate cysteine as opposed to linkage of two chains.[1]

Experimental Procedures

Materials. Saccharomyces cerevisiae cytochrome *c* type VIII was purchased from Sigma. Preparations of the protein were subjected to exclusion chromatography at 25 °C using a calibrated 2.2 × 65 cm column of Sephadex G-50 equilibrated with either 50 mM phosphate buffer, pH 7.0, or 50 mM

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[1] The sequence numbering system employed assigns the two cysteine residues linked by thioether bonds with the porphyrin to sequence positions 14 and 17.

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