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FOR THE RECORD

Volume changes of the molten globule transitions of horse heart ferricytochrome *c*: A thermodynamic cycle

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Abstract: Volume changes among the unfolded (U), native (N), and molten globule (MG) conformations of horse heart ferricytochrome *c* have been measured. U to N (pH 2 to pH 7) was determined in the absence of added salt to be -136 ± 5 mL/mol protein. U to MG (pH 2, no added salt to pH 2, 0.5 M KCl) yielded $+100 \pm 6$ mL/mol. MG to N was broken into two steps, N to NCl_X at pH 7 by addition of buffered KCl to buffered protein lacking added salt (NCl_X = N interacting with an unknown number, X, of chloride ions), and MG to NCl_X by jumping MG at pH 2 in 0.5 M KCl to pH 7 at the same salt concentration. The ΔV of N to NCl_X was -30.9 ± 1.4 mL/mol protein, whereas MG to NCl_X entailed a ΔV of -235 ± 6 mL/mol. Within experimental error, the results add up to zero for a complete thermodynamic cycle. We believe this to be the first volumetric cycle to have been measured for the conformational transitions of a protein. The results are discussed in terms of hydration contributions from deprotonation of the protein, other hydration effects, and the formation and/or enlargement of packing defects in the protein's tertiary structure during the steps of folding.

Keywords: cytochrome *c*; hydration; internal packing; molten globule; volume change

Considerable interest has arisen in recent years in non-native conformational states of proteins that are not fully unfolded in the sense of being random coils. Among these the "molten globule" (for reviews see Kuwajima, 1989; Christensen & Pain, 1991; Ptitsyn, 1992; Haynie & Freire, 1993) has received considerable attention. Because protein folding often follows apparent two-state kinetics, intermediates are difficult to study owing to the fact that they are usually present at vanishingly small concentrations and then only briefly. The MG is particularly interesting because it appears to be both a thermodynamically stable conformational state and, at least for some proteins, a kinetic intermediate on the folding pathway (Dolgikh et al., 1984). In addition, there is evidence that MG-like conformations may be involved in chaperone-mediated folding in vivo (reviewed by Martin & Hartl, 1993) and in translocation across membranes (Bychkova et al., 1988).

In its purest form, the MG has essentially native secondary structure but a fluid, fluctuating tertiary structure. Although compact, it is not as compact as the native form, but neither the nature of the packing nor the degree of solvent exposure is known. Because changes in volume reflect changes in hydration and in packing, they are a particularly good probe of conformational transitions. We have therefore measured the volume changes among the unfolded, native, and MG forms of horse heart ferricytochrome *c*, obtaining a complete thermodynamic cycle. We believe this to be the first such cycle to have been measured.

Cytochrome *c* is native at pH 7. At pH 2, electrostatic repulsions caused by the large net positive charge of +24 (Stellwagen & Babul, 1975; Shaw & Hartzell, 1976) lead to the unfolded form. Addition of salt to U screens the charges and allows collapse to MG (Goto et al., 1990; Goto & Nishikiori, 1991; Kuroda et al., 1992). The transition from MG to N entails a change in both pH and salt concentration. Measurement of protein-free buffer controls in this case is technically inconvenient. The triangle linking U, MG, and N was therefore expanded to a square in which the transition between N and MG was divided into two steps. In one, the native form in buffer at pH 7 but in the absence of added salt was mixed with pH 7 buffer containing KCl to a final salt concentration of 0.5 M. This produces a state which we call NCl_X, the native conformation that interacts with an unknown number, X, of chloride ions. The second step converts MG to NCl_X by jumping the pH from 2 to 7 at a constant KCl concentration of 0.5 M. The logic is shown in Figure 1.

Horse heart cytochrome *c* was purchased from Sigma Chemical Co. (St. Louis, Missouri; type VI, lot 62H7115). It was used without further purification. Neither volumetric nor spectroscopic measurements differed between lots. MOPS was from

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Abbreviations: U, unfolded; N, native; MG, molten globule; NCl_X, native form interacting with an unknown number, X, of chloride anions.

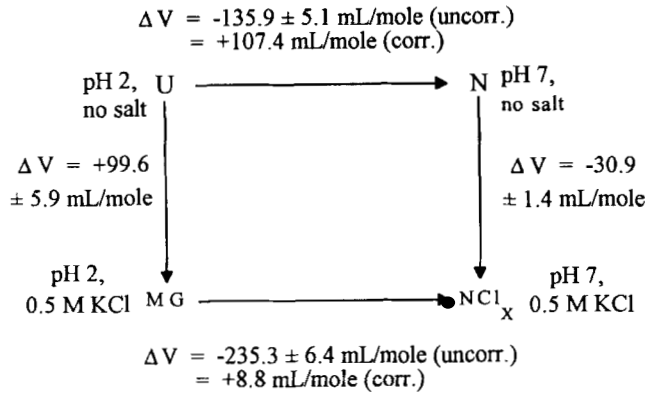

Fig. 1. Conformational transitions of horse heart ferricytochrome *c* and the volume changes associated with them. Volume changes are given in mL/mol of protein ± standard errors of the mean. In a typical experiment, the U to N and U to MG transitions were measured at the same time on aliquots of the same protein solution. Into one leg of the dilatometer, 3.00 mL of 13.1 mg/mL protein were loaded. For U to N, 2.50 mL of 0.1 M MOPS buffer at pH 7.81 were placed in the other leg. For U to MG, 2.50 mL of 1 M KCl whose pH had been adjusted to 2.00 with HCl were loaded into the other dilatometer leg. The transition from MG to NCl_X was performed on 3.00 mL of protein, 16.1 mg/mL, in HCl at pH 2.00 containing 0.5 M KCl. This was mixed in the dilatometer with 3.70 mL of 0.1 M MOPS buffer, pH 7.50, containing 0.5 M KCl. In the N to NCl_X reaction, 3.00 mL of native protein at 14.9 mg/mL in 0.1 M MOPS at pH 7.00 were mixed with 2.80 mL of 1 M KCl whose pH had been adjusted to 7.00 with 1 M KOH.

U.S. Biochemical (Cleveland, Ohio). All other chemicals were of the best grade commercially available. The building's distilled water supply was passed through demineralizing, charcoal, and submicron filters. Its conductivity did not exceed 10^{-6} ohm^{-1}.

pH measurements were performed with a Radiometer PHM 64 pH meter equipped with a Radiometer S300 combination electrode. The instrument was standardized at pHs 7 and 4 with standard buffers (Fisher) before all manipulations and checked for drift after measurement with pH 7 standard buffer. The asymmetry potential was never less than 90%. The error in pH measurement is ±0.02 pH units.

Protein concentrations were determined spectrophotometrically at the Soret absorption peak by diluting a carefully measured aliquot into 0.1 M MOPS buffer, pH 7.0, in a 10-mL volumetric flask and making up to volume with the same buffer. An extinction coefficient of 8.64 (cm-mg/mL)$^{-1}$ (Babul & Stellwagen, 1972) was used. Absorption spectra were measured in matched 1-mm cells on a Cary 17D recording spectrophotometer against MOPS buffer.

For experiments in the U to N direction, the protein was dissolved in 10 mM HCl and dialyzed overnight at 4 °C against the same solvent. For experiments in the MG to NCl_X direction, the 10 mM HCl also contained 0.5 M KCl. The pH after dialysis was usually slightly higher than 2 and was adjusted to pH 2.00 with a small amount of 1 M HCl for U and 1 M HCl containing 0.5 M KCl for MG. Native cytochrome *c* for N to NCl_X experiments was made up in and dialyzed against 0.1 M MOPS, pH 7.00, at 4 °C. Its pH was checked afterward.

Solutions were loaded into the dilatometers with high-precision syringe microburettes (Micrometric Instrument Co., Tampa, Florida) calibrated to dispense 5 μL per scale division. The same microburettes were used for titrations at the pH meter. Volumetric measurements were performed at 20 °C in modified Carlsberg dilatometers as described (Ybe & Kahn, 1994). The capillaries were individually calibrated (Kahn & Briehl, 1982) and measured 1.130, 0.499, 0.489, and 0.486 μL/cm change in heptane level. The changes in heptane height in individual experiments ranged from approximately 1 to 3 cm. The protocol for dilatometric experiments involves placing the protein solution in one leg of the unit. The other leg is loaded with a solution that, when mixed with the protein, induces the conformational transition. Thus, for the U to MG transition, for example, protein at pH 2.00 would be loaded into one leg and KCl solution whose pH had been adjusted to 2.00 would be dispensed into the other (see also the legend to Fig. 1). Because the volume changes of some of the transitions are dependent in a complex way on both the pH and the protein concentration (concentration dependence does not appear to arise from aggregation; S. Spector, K. Foygel, & P.C. Kahn, unpubl.; see also Trewhella et al., 1988), care was taken to ensure (1) that the final protein concentration, 7.2 mg/mL, was the same in all experiments, and (2) that the initial and final pHs were set to 2.00 and 7.00, respectively. In addition, the initial protein concentrations were kept within a reasonably narrow range of 13–16 mg/mL (see below). For all experiments, concurrent control dilatometers were run in which the protein solutions were replaced by equal volumes of the appropriate dialysis buffer against which the protein had been equilibrated. The volume changes observed in the control dilatometers were subtracted from the corresponding protein-containing units.

Because the initial protein concentrations varied from 13 to 16 mg/mL, controls to measure the volume change of protein dilution between these concentrations were performed on the MG form. The volume change was zero within an experimental error of ±2.5 mL/mol of protein. Volume changes of protein dilution between 13 and 7.2 mg/mL were also zero.

The pH jump in the U to N and MG to NCl_X reactions removes protons from the protein. Volume changes of this proton removal are not, of course, included in the buffer controls described above. Cytochrome *c* contains 3 Asp, 9 Glu, 2 heme, and the α-carboxyl, for a total of 15 carboxyls. Removal of a proton from each of these entails a volume change of −11 mL/mol of carboxylate formed (Rasper & Kauzmann, 1962), or $-11 \times 15 = -165$ mL/mol of protein titrated. There are three histidines. Deprotonation of these yields +2.1 mL/mol of imidazole as deduced from Rasper and Kauzmann (1962). In the absence of added salt, all three titrate between pH 2 and pH 7 for a volume change of +6.3 mL/mol of protein. In the presence of salt, however, the pK_a of His-18, one of the axial ligands of the heme iron, is shifted to lower values (Stellwagen & Babul, 1975; Dyson & Beattie, 1982) and only two titrate, generating +4.2 mL/mol of cytochrome. Deprotonation thus occurs with an overall volume change of $-165 + 6.3 = -158.7$ mL/mol of protein in the absence of added salt and $-165 + 4.2 = -160.8$ mL/mol in its presence.

The protons thus removed protonate the MOPS buffer. The volume change of protonating MOPS was therefore measured by bringing 12 mL of 0.1 M MOPS from pH 7.5 to pH 7.0 in a dilatometer with the appropriate volume of 0.01 M HCl. Parallel dilution controls were run and subtracted. From the initial and final pH values and the pK_a of 7.5, the resulting volume change could be calculated from the raw data and was −4.9 ± 2.5 mL/mol of MOPS titrated. The transfer of 18 mol of H^+

to the buffer in the absence of added salt thus involves $-4.9 \times 18 = -88.2$ mL/mol of cytochrome *c*. Protonation of 17 mol of MOPS in the presence of added salt entails $-4.9 \times 17 = -83.3$ mL/mol of protein. This yields overall values for deprotonation of the protein and protonation of the buffer of −246.9 and −244.1 mL/mol of cytochrome *c* at low and high salt concentrations, respectively.

Results and discussion: The results are shown in Figure 1, in which the arrows indicate the direction in which the measurement was performed and for which signs of the volume changes are given. The values labeled "uncorrected" are those obtained after subtraction of the protein-free buffer controls but before subtraction of the volume changes due to deprotonation of the protein and transfer of the resultant protons to the buffer. Subtraction from these of −246.9 mL/mol of protein in the absence of added salt and of −244.1 mL/mol in its presence yields the corrected values, which thus represent the sum of all contributions to the volume change other than deprotonation of the protein and transfer of its protons to the buffer.

The changes for the legs of a complete thermodynamic cycle must add up to zero. The sum of the results in Figure 1 in the clockwise direction is −31.1 mL/mol of protein. This is only 6.2% of the summed absolute values of the four legs. In addition, standard methods for the propagation of error for the four legs of Figure 1 yield a standard error of the mean for the cycle of ±10.2 mL/mol. The 95% confidence limits (*t*-test, 3 degrees of freedom) are ±32.5 mL/mol, which, when added to −31.1 mL/mol, includes zero. The null hypothesis, that the sum of the legs equals zero, is therefore accepted with 95% probability. The requirement of the cycle is thus met.

The N to NCl_X ΔV arises from chloride binding to the native form, whose conformation is unaffected by 0.5 M KCl. This was checked by circular dichroism spectroscopy (data not shown). Because of the relatively high pI = 10.04 of cytochrome *c*, the protein carries a net positive charge at pH 7 ($Z \approx +7.1$ at pH 7.15 and $\mu = 0.1$ [Shaw & Hartzell, 1976]). This would give rise to an electrostatic attraction for anions, and the protein is known to bind anions, including chloride (Stellwagen & Babul, 1975; Taborsky & McCollum, 1979; Osherhoff et al., 1980). Electrostatic interactions would release electrostricted water, which would cause a net rise in volume (Cohn et al., 1934; Cohn & Edsall, 1943). ΔV is negative, however, so if release of electrostricted water occurs, its effect is masked by another. Another possibility is that the change in ionic strength alters one or more pK_a values of the protein's titratable groups, leading to a change in protonation at neutral pH that we have not taken into account. More likely is a reduction in the size and/or number of packing defects. Trewhella et al. (1988) have shown by means of small-angle X-ray scattering that addition of salt to native cytochrome reduces the radius of gyration by approximately 1 Å (see also Liu et al., 1989). At neutral pH, the protein's net charge would subject it to a degree of electrostatic stress. Screening of the charge would reduce the stress, allowing the structure to relax and shrinking the total packing defect volume.

The rise in volume for U to MG has two possible sources. The first is expulsion of some water of hydration from the protein surface as the relatively more extended U form collapses to the more compact MG, a collapse brought about by the ionic strength induced reduction in electrostatic repulsions (Goto & Nishikiori, 1991). Expulsion of water from aliphatic surface occurs with a rise in volume (Kauzmann, 1959; Friedman & Scheraga, 1965), whereas the withdrawal of aromatics from solvent contact entails a fall (Kasarda, 1970; Weber et al., 1974; Li et al., 1976; Visser et al., 1977). Because there is considerably more aliphatic surface than aromatic, a net rise from hydration changes is likely. The second is the formation or enlargement of packing defects—void spaces—in the course of the transition (Kauzmann, 1959). Formation or enlargement of voids also involves a rise in volume. Being compact but not as well packed as N, the MG form probably contains void space not present in U, so both hydration and packing defects together contribute to the ΔV.

MG to NCl_X, of course, involves the same contribution from deprotonation as does U to N. It is interesting that when this contribution is subtracted out, the remaining volume change, +8.8 mL/mol, is quite small. If, as suggested above, the volume of voids within the protein is smaller in NCl_X than in N, the contribution of packing in MG to NCl_X would be small. Because MG is nearly as compact as N, most of the water of hydration that is going to depart from the surface of U in the course of forming the native structure has done so by the time MG is formed. Although some contribution from hydration is undoubtedly present in MG to NCl_X, it would be smaller than that of U to MG. There is evidence that the heme pocket is not tightly closed in the MG form (Stellwagen & Babul, 1975; Dyson & Beattie, 1982; Goto et al., 1990; Myer & Saturno, 1991; Goto & Nishikiori, 1991; Kuroda et al., 1992), so some desolvation of the heme and, possibly, of other aromatics, would yield a small volume decrease. Burial of some aliphatic surface would cause a small increase. A variety of oppositely signed small factors would thus seem to account for the result.

The high-resolution crystallographic structure of ferricytochrome *c* (Bushnell et al., 1990; Protein Data Bank file 1HRC, Bernstein et al., 1977) contains 124 water molecules per molecule of protein. Seven of these have accessible surface areas of zero as calculated by the method of Lee and Richards (1971). The average volume of these buried waters (Richards, 1974) is 28.5 ± 5.1 $Å^3$ (SD; SEM is 1.9 $Å^3$). The volume occupied by a water molecule in the macroscopic phase as computed from the molecular weight of 18 g/mol and specific volume of 1 mL/g is 30 $Å^3$, so these waters occupy volumes essentially the same as those of bulk water molecules. Their burial could only contribute significantly to the volume changes measured here if their volumes while within the hydration zone of U were significantly different from 28.5 $Å^3$. A difference of 1 $Å^3$ per molecule of water would be 0.6 mL/mol of solvent. Seven such waters would yield 4.2 mL/mol of protein, which is within the error of the experiments. A 1-$Å^3$ difference in density of the water of hydration would be 3% of the bulk density, so these seven buried waters would have to differ from bulk by more than this for their contributions to be significant.

A crude estimate of the number of water molecules involved in the nonelectrostatic component of U to N can be made. To do so one must assume a density of this water when it hydrates the surface of U before being expelled upon folding into the bulk solution, where the density is 1 g/mL. Water being a relatively incompressible liquid, an average density difference of 1% would yield a volume change per molecule of 0.3 $Å^3$ or 0.18 mL/mol of water expelled. The ΔV of U to N in Figure 1 after correction for proton titration is +107 mL/mol of protein. The number of waters expelled by this calculation is thus $107/0.18 = 594$. This number would be lower if the density difference were larger. It must also be reduced by the contribution made by the

formation of packing defects to the process, a contribution that cannot, at present, be evaluated, but which could be significant. The expulsion of ~600 molecules of water in U to N is therefore an upper limit. U to MG would expel somewhat fewer waters from the protein surface, and MG to N or NCl_X fewer still.

Depending on the solution conditions, cytochrome *c* appears to adopt a number of non-native states (Ohgushi & Wada, 1983; Myer & Saturno, 1990, 1991; Jeng & Englander, 1991; Kuroda et al., 1992; however, see also Goto et al., 1993). Other proteins may well do likewise, although there is no reason to believe a priori that all of them will do so. A fuller exploration of the volumetric behavior of cytochrome *c* is in progress. Lee (1991) has shown that the change in solvent-accessible surface area upon denaturation is roughly half the difference in areas between the native and fully extended forms. In view of the fact that the energetics of protein folding appear to be proportional to the surface area (Hermann, 1972; Chothia, 1974; Eisenberg et al., 1986), it is likely that other extensive thermodynamic properties will also prove to be linearly proportional to changes in accessible surface area. It would be useful if relatively simple volumetric measurements could be used to determine actual changes in exposed surface area. It would also be useful if such relationships could distinguish hydration effects from those due to changes in the size and/or number of packing defects. This, too, is in progress.

We believe the measurements presented here to be the first complete thermodynamic cycle of the volumetric changes associated with the conformational transitions of a protein. They provide insight into the relative roles of protonation and nonprotonation-related factors in the molten globule transitions of cytochrome *c*, and they allow us to begin, at least, to assess the roles of hydration and packing.

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