The Contribution of Electrostatic Factors to the Stabilization of the Conformation of Cytochrome c

STUDIES ON THE MALEYLATED PROTEIN

(Received for publication, September 21, 1978)

Abel Schejter, Miri Zuckerman, and Irit Aviram

From the Department of Biochemistry, George S. Wise Center for Life Sciences, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel

All the lysines of horse heart cytochrome c were maleylated yielding a low spin product. At room temperature and low salt concentration, this product lacked the 695 nm absorption band and showed tryptophan fluorescence and circular dichroic spectra typical of denatured cytochrome c. The 695 nm band and the native tryptophan fluorescence and circular dichroic spectra were restored by addition of salts, their effectiveness being dependent on the charge of the cation. On low salt concentration, the 695 nm band was also restored by lowering the temperature. Studies of the temperature dependence of the 695 nm band indicate that the thermal denaturation of maleylated cytochrome c occurs at temperatures 60-70°C lower than in the native protein. This implies a destabilization of the native conformation by 5.6 kcal/mol; a similar value is evidenced by comparative urea denaturation studies on the native and modified proteins. The results confirm the assumption that the native conformation of cytochrome c is mostly determined by interactions involving internal residues.

Mammalian type cytochromes c are highly charged molecules, with pI values around 10. Horse cytochrome c, for example, is a strong cation at pH 7, with a net charge of 9.5 resulting from 22.5 positively charged and 13 negatively charged groups (1).

The native folding of the protein chain at neutral pH is the resultant of several types of forces: hydrogen bonds, hydrophobic, short range and long range van der Waals' interactions, and electrostatic attractions and repulsions. The fact that this native folding is highly conserved during evolution (2) emphasizes its important role in determining the properties of all c-type cytochromes.

The electrostatic interactions take place between side chains distributed over the protein surface. In principle, it is possible, therefore, to introduce perturbations into the electrostatic contribution to the conformation of the protein by suitable modification of the charged residues, without affecting the forces that operate in the interior domain of the molecule. This can be achieved, for example, by modifying the ε-amino residues of horse cytochrome c.

Several extensive modifications of lysyl residues of cytochrome c involving changes in their electrostatic charge have been reported. In the case of acetylation, which included blocking of tyrosyl groups, the product had a high spin-type spectrum (3). Trifluoroacetylation and deamination (4) did not change the low spin-type spectrum of the molecule. These studies were mainly concerned with the effects of the modification of lysyl residues on the interactions between cytochrome c and physiological oxidants and reductants, but the effects on the conformation of the protein were not studied extensively. In the present study we report the effects of full maleylation of the lysines of horse cytochrome c on the conformation of the protein, and show that these effects can be reversed by proper manipulation of the temperature and electrolyte composition of the medium.

EXPERIMENTAL PROCEDURES

Horse heart cytochrome c (Sigma Chemical Co., type III) was purified by ion exchange chromatography (5). All reagents employed were of best available analytical grade.

Methods—Optical absorption spectra were recorded with a Cary model 118 spectrophotometer. Fluorescence spectra were obtained using a Hitachi-Perkin-Elmer spectrofluorimeter. Circular dichroism spectra were measured in a Cary model 60 spectropolarimeter; molar ellipticities were estimated by the formula [8] = 2,303 (4500/π) (εL - εS) and expressed in degrees cm² per dmol of protein.

Molecular weights were estimated by equilibrium sedimentation measurements, carried out in Beckman model E analytical ultracentrifuge. Absorbance readings were made at 410 and 280 nm and expressed as the pen deflection of the recorder.

Rapid kinetic data were measured in a Gibson-Durrum stopped flow spectrophotometer.

Proton nmr spectra were kindly obtained by Professor Kurt Wüthrich from Eidgenössische Technische Hochschule, Zurich.

Preparation and Chemical Analysis of Maleylated Cytochrome c—Cytochrome c was maleylated by the method of Butler et al. (6) and free lysines were estimated by reaction with trinitrobenzenesulfonate (7). Details of these procedures were published elsewhere (8). None of the preparations used in this study contained more than 0.4 mol of lysines/mol of protein.

RESULTS

The visible optical spectrum of maleylated ferric cytochrome c at 25°C and in dilute buffers is very similar to that of the native protein, with two exceptions: the Soret absorption peak is more intense and blue-shifted by 2 nm and the 695 nm band is absent. The absence of the 695 nm band is an indication that introduction of 19 maleyl residues, involving a net charge of -38 electrostatic charges, resulted in breakage of the iron-sulfur bond (2).

In order to establish that the changes introduced by the modification were not confined to the heme region, fluorescence and circular dichroism spectra of the product were also recorded.

It is well known that the fluorescence of the tryptophanyl residue of cytochrome c may be detected only when major conformation changes, such as denaturation, displace the flu-
Electrostatic Forces and Cytochrome c Conformation

orophore from its normal position with respect to the heme group (9, 10). The emission spectra of maleylated cytochrome c (Fig. 1) indicate that such changes did occur, since the yield of the fluorescence amounted to 80% of the fluorescence of fully denatured ferricytochrome c in 6 M guanidine HCl.

A similar conclusion is borne out by the circular dichroism spectrum of maleylated cytochrome c, shown in Fig. 2. This spectrum is strikingly similar to that of denatured cytochrome c (11).

The expected molecular weight of fully maleylated cytochrome c is about 14,200. In equilibrium sedimentation experiments, a molecular weight of 17,300 was estimated, based on the plot shown in Fig. 3 and assuming $V = 0.725$ (1). Although this is about 20% higher than expected, the linearity of the plot indicates that no aggregation occurs in the system; thus, the observed properties of the modified molecule cannot be attributed to the formation of dimers or polymers.

The properties described above are characteristic of maleylated cytochrome c at 25°C and in solutions of low ionic strengths, and change markedly upon addition of salts or cooling. Thus, it was found that the 695 nm band could be restored by addition of neutral salts. The effectiveness of the salts in restoring the band is: $NaCl < Na_2SO_4 < MgCl_2$ which indicates that it does not depend only on the ionic strength effect due to the salts, but rather on the concentration and charge of the cation (Fig. 4). Thus, at 20°C and in 0.02 M phosphate buffer, pH 7.0, 50% restoration of the band is achieved at $6 \times 10^{-4}$ M MgCl$_2$ or $3.5 \times 10^{-2}$ M NaCl.

It should be noticed that at the concentration of MgCl$_2$ corresponding to half-saturation the molar concentration of the ligand is only 5 times higher than that of the protein. Concomitantly with the reappearance of the 695 nm band, tryptophan fluorescence undergoes quenching (Fig. 1) and the circular dichroism spectrum becomes similar to that of native ferricytochrome c (Fig. 2). The high field nmr spectrum of the protein under these conditions (Fig. 5) shows a resonance at
23.8 ppm (relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate) that is indicative of methionine coordination to the iron (12).

Kinetics of the salt-induced spectral changes could be followed spectrophotometrically by recording the rate of decrease of intensity at the Soret peak after rapid mixing of solutions of maleylated cytochrome c in dilute buffer with different concentrations of NaCl and MgCl₂. The reaction was of first order with respect to cytochrome c and the observed rate constants increased linearly with the salt concentration (Fig. 6). An activation energy of 14.7 kcal/mol was estimated for the conformation changes measured in 10⁻² M MgCl₂ in the temperature range 10–37°C.

Thus, maleylated cytochrome c in dilute buffer at neutral pH resembles denatured, low spin cytochrome c. Neutral salts induce conformational changes leading to a native-like molecule. The same process can be caused by cooling in the absence of salts. In Fig. 7, the change in absorbance of ferric maleylated cytochrome c at 695 nm in 0.02 M phosphate buffer, pH 7.0, is shown as a function of temperature. Qualitatively, the results are very similar to those observed for the native protein, which loses its 695 nm band at high temperatures (13, 14). In maleylated cytochrome c this transition occurs at significantly lower temperatures. When the spectral transition is treated as an equilibrium between denatured (hot) and native (cold) forms of the protein (13), it is possible to compare the thermodynamic parameters of this equilibrium in the native and modified proteins. The equilibrium constant at each temperature is estimated from the ratio (A - A₀)/(A₀ - A) where A₀ is the absorbance of the native-like (cold) maleylated cytochrome c measured in the presence of 10⁻² M MgCl₂, A₀ is the final absorbance obtained upon heating and corresponding to the denatured (hot) protein, and A is the absorbance measured at a particular temperature. Fig. 8 shows the plot of log Kₑᵥₑ versus 1/T for maleylated cytochrome c, and Table I lists the entropies and enthalpies attending this equilibrium for the native and the maleylated protein.

In temperature dependence experiments, it was observed that higher concentrations of maleylated cytochrome c shifted the equilibrium towards the closed form. Fivefold dilution of the protein from 4.7 × 10⁻⁴ M to 9 × 10⁻⁵ M increased the equilibrium constant between the hot and cold forms estimated at 25°C from 1.17 to 9.8 without affecting ΔH (Fig. 8). A similar shift could be produced by varying the concentration of phosphate buffer from 0.05 to 0.02 M, indicating that the protein contribution to the ionic strength stabilizes the native-like form of maleylated cytochrome c.

It was of interest to compare the closed form of maleylated cytochrome c saturated with MgCl₂ with the native molecule in terms of their stability in the presence of denaturants. This was done by following the dependence of the 605 nm band on the concentration of urea (Fig. 9). The denaturation visualized by bleaching of the absorption peak occurred at very low concentrations of urea. The data were analyzed in terms of a two-state mechanism (15, 16). The apparent equilibrium constant, Kₑᵥₑ between the denatured and native-like forms was
calculated using:

$$K_D = \frac{A - A_N}{A_N - A}$$  \hspace{1cm} (1)

where $A$ is the observed absorbance at 695 nm, and $A_N$ and $A_D$ represent absorbances of the native-like and fully denatured states of maleylated cytochrome c. $\Delta G_D$, the apparent free energy of denaturation, calculated from $K_D$ values, was plotted (Fig. 9) against the concentration of urea according to the equation:

$$\Delta G_D = \Delta G_{D, 0} - m(D)$$  \hspace{1cm} (2)

This permitted evaluation of the free energy of unfolding of the closed form of maleylated cytochrome in the absence of denaturant and comparison with the corresponding value obtained for native cytochrome c. $\Delta G_{D, 0}$ of 1.88 kcal/mol was estimated; this value, compared to the value of 7.44 kcal/mol calculated from the results reported by Tsong for native cytochrome c (17, 18) indicates a drastic decrease in stability.

$\begin{array}{|c|c|c|c|}
\hline
\text{State} & \Delta G & \Delta H & \Delta S \\
\hline
\text{Native cytochrome c} & +2.2 & +19.8 & +67 \\
\text{1st step} & & & \\
\text{Native cytochrome c} & +4.3 & +45.1 & +133 \\
\text{2nd step} & & & \\
\text{Maleylated cytochrome c} & -1.34 & +41.5 & +144 \\
\hline
\end{array}$

$^a$ Determined in 0.05 M phosphate buffer pH 7.0 (14).

$^b$ Determined in 0.02 M phosphate buffer, pH 7.0; the concentration of protein was $9 \times 10^{-5}$ M.

**DISCUSSION**

One of the characteristics of mammalian cytochromes c is their high cationic charge (1). The requirements of positive charges for the normal interaction between cytochrome c and the mitochondrial membrane and its components are well documented (19); the effects of electrostatic interactions on the oxidation-reduction properties of horse cytochrome c are also known (20). The aim of the present study was to clarify to what extent the electrostatic charge of the molecule contributes to the stability of its native state, characterized by the coordination of iron to histidine-18 and methionine-80. Charged groups are usually located on the molecular surface of proteins; blocking of the lysine-positive groups of cytochrome c by negatively charged groups should ensure preservation of the orientation of these side chains toward the exterior. Any change in the interior of the molecule would then originate mainly in destabilization arising from electrostatic repulsions between the negatively charged residues spread over the modified molecule.

In native horse cytochrome c, the net charge of the protein at neutral pH is about $+9$ (1); this net charge is the result of 22 positive charges and 13 negative charges. Thus, both electrostatic attractions and repulsions act in the stabilization of the native conformation of the molecule. In fully maleylated horse cytochrome c, the overall net charge can be estimated to be $-30$, resulting almost exclusively from negative charges; thus, the attractive forces have been eliminated and many
new repulsive forces have been introduced. A profound destabilization of the active conformation should therefore be expected. Indeed, maleylated cytochrome c in dilute buffer solutions in many respects resembles denatured cytochrome c: the iron-methionine bond is broken, as evidenced by loss of the 695 nm band; the circular dichroism is similar to that reported by Myer for denatured cytochrome c (11) and the quenching effect of heme on the fluorescence of tryptophan-side chains is diminished. The property of identity of the sixth ligand in the maleylated protein was discussed elsewhere (8).

The ability of neutral salts to restore the iron-methionine bond and the globular native-like structure, evidenced by the reappearance of the 695 nm band and reversal of the fluorescence and circular dichroism spectra (Figs. 1 and 2), can be attributed to their shielding effects (21, 22). The high effectiveness of MgCl₂ compared to Na₂SO₄ or NaCl suggests that salts exert their action mainly through the electrostatic interactions between their cations and the multiple anionic groups of the modified protein. At half-saturation with MgCl₂, the concentration of the ligand is 6 × 10⁻⁴ M compared to 1.25 × 10⁻⁴ M concentration of the protein, or 23.75 × 10⁻⁷ M concentration of maleyl side chains (Fig. 4). The implication of this stoichiometric relationship is that in order to restore the native structure of the maleylated protein it is not necessary to cancel all its charges. A possible explanation of this fact is the assumption of multidentate chelation of the cation by the modified protein carboxyl groups. This, however, would introduce structural constraints that would hinder the restoration of the native conformation. The assumption of monodentate binding, on the other hand, leads to different possibilities. Thus, for example, the native folding may be incompatible with overall electrostatic charges that are beyond certain critical values. There may also exist specific electrostatic repulsions at specific sites that prevent the achievement of the native folding, and are relieved in the presence of cations.

A better understanding of the factors responsible for the salt-induced restoration of the native conformation requires the examination of partially maleylated derivatives of horse cytochrome c; this is now in progress.

Maleylated cytochrome c shows an interesting temperature-dependent behavior: in dilute buffer solutions, upon cooling, the spectroscopic properties typical of the native conformation are restored. Native cytochrome c does not show spectroscopic temperature-dependent changes below 25°C; at higher temperatures its conformation changes in a spectroscopically operable two-step process (14). The first step starts at about 35°C; it involves the displacement of methionine-80 from iron coordination and small changes in tertiary structure (24). The analysis of this step as a two-state equilibrium and using the van't Hoff equation (14) leads to the thermodynamic parameters listed in Table I. The second step beginning at about 70°C is a helix-coil transition (24) that is accompanied by a large absorption of heat (25), characteristic of protein denaturation (26). If this step, too, is analyzed as a two-state equilibrium and assuming van't Hoff behavior, quite different thermodynamic parameters are obtained (Table I) (14).

For maleylated cytochrome c in dilute buffer, the stability of the native-like conformation is lost at much lower temperatures. Its analysis in terms of the van't Hoff equation leads to heats and entropies that are strikingly similar to those observed for the second step of the thermal denaturation of native cytochrome c. Thus, at 25°C the estimated free energy changes for the thermal denaturation of native and maleylated cytochrome c differ by 5.6 kcal/mol (see Table I). The modified protein being the less stable. This results in a shift of the transition temperature of 60-70°C. Undoubtedly, a proper quantitative analysis of the thermal stability of maleylated cytochrome c must await a direct calorimetric determination, because there is no certainty that the assumptions implicit in the use of the van't Hoff equation apply to this case. It is noteworthy, however, that comparison of the free energies of unfolding by urea of native and maleylated cytochrome c, estimated from Equation 2, also yields a difference of 5.6 kcal/mol.

The native state of proteins is often referred to as being one of marginal stability. The fact that the effects of such a drastic disruptive modification of the electrostatic interactions between charged groups located in the molecular surface can be relieved by low concentrations of cations or by a slight shift in temperature indicates that the native conformation of cytochrome c is mostly determined by interactions involving internal residues.

The chemical reactivity of native cytochrome c is strongly dependent on the strength of the iron-protein ligand bonds; these, in turn, depend on the integrity of the native conformation. Furthermore, the reactivity of native cytochrome c is affected by its electrostatic charge. Since the stability of the iron-methionine-80 sulfur bond of maleylated cytochrome c can be altered by small changes in tertiary structure (24) and by changes in temperature, this modified molecule is a useful model for quantitative study of relationships between conformation, electrostatic charge, and reactivity parameters. The latter includes the very important kinetic and thermodynamic parameters of oxidation-reduction reactions. Studies now in progress are expected to yield relevant information on this problem.

REFERENCES
1. Margoliash, E., and Schejter, A. (1966) Adv. Protein Chem. 21, 113-283
2. Dickerson, R. E., and Timkovitch, R. (1975) in The Enzymes (Boyer, P. D., ed) Vol. 11, pp. 397-547, Academic Press, New York
3. Wada, K., and Okunuki, K. (1969) J. Biochem (Tokyo) 66, 263-272
4. Fanger, M. W., and Harbury, H. A. (1965) Biochemistry 4, 2541-2545
5. Margoliash, E., and Walasek, O. (1967) Methods Enzymol. 10, 333-348
6. Butler, P. J. B., Harris, Y. I., Hartley, B. S., and Leberman, R. (1969) Biochem. J. 112, 679-688
7. Habebe, A. F. S. A. (1960) Anal. Biochem. 14, 328-330
8. Pettigrew, G. W., Aviram, I., and Schejter, A. (1976) Biochem. Biophys. Res. Commun. 68, 807-813
9. Weber, G., and Teale, W. J. (1969) Disc. Farad. Soc. 27, 134-141
10. Tsong, T. Y. (1974) J. Biol. Chem. 249, 1988-1990
11. Myer, Y. P. (1968) Biochemistry 7, 765-776
12. Wuthrich, K. (1969) Proc. Natl. Acad. Sci. U. S. A. 63, 1071-1078
13. Schejter, A., and George, P. (1964) Biochemistry 3, 1045-1049
14. Pettigrew, G. W., Aviram, I., and Schejter, A. (1975) Biochem. J. 149, 155-167
15. Pace, C. N. (1975) CRC Crit. Rev. Biochem. 3, 1-43
16. Tanford, C. (1968) Adv. Protein Chem. 23, 122-280
17. Aviram, I., and Weissmann, C. (1978) Biochim. Biophys. Acta 14, 1542-1547
18. Tsong, T. Y. (1975) Biochim. Biophys. Acta 149, 154-199
19. Ferguson-Miller, S., Brautigan, D. L., and Margoliash, E. (1978) J. Biol. Chem. 253, 149-159
20. Margalit, R., and Schejter, A. (1973) Eur. J. Biochem. 12, 492-499
21. Von Hippel, P. H., and Schleich, T. (1968) in Biological Macromolecules (Timasheff, S., and Fasman, G., eds) Vol. 2, pp. 417-573, Marcel Dekker, New York
22. Jacobson, A. L. (1963) Biopolymers 1, 269-276
23. Schejter, A., and Aviram, I. (1969) Biochemistry 8, 1491-153
24. Urey, D W (1965) Proc. Natl. Acad. Sci. U. S. A. 54, 640-648
25. Privalov, P. L., and Khechinashvili, N. N. (1974) J. Mol. Biol. 86, 655-684
26. Jackson, W M., and Brandts, J. F. (1970) Biochemistry 9, 2294-2301
The contribution of electrostatic factors to the stabilization of the conformation of cytochrome c. Studies on the maleylated protein.
A Schejter, M Zuckerman and I Aviram

J. Biol. Chem. 1979, 254:7042-7046.

Access the most updated version of this article at http://www.jbc.org/content/254/15/7042

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/15/7042.full.html#ref-list-1