The Insensitivity of the 695 nm band of horse heart ferricytochrome c to protein conformation.

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Summary: Changes in extinction of horse ferricytochrome c bands around 285, 290, 410, 530, 655 and 695 nm in solutions at pH 7 and 25°C were measured by difference spectrophotometry for increasing concentrations of guanidine hydrochloride (I) and other conformation perturbants. For I $C_{\frac{1}{2}}$ values for the changes at 285, 290, 655, & 695 nm lie between 2.5 and 2.6M, but at 530 nm, 3.1M. The magnitude of the changes at 285 and 290 are consistent with the exposure of TYR-48 & -67, and TRP-59 to the solvent. The co-incidence of $C_{\frac{1}{2}}$ for 695 and the protein bands indicates conformation insensitivity. With 0.159M CN^- the 695 nm band disappears but not more than 2% exposure of TRP & TYR occurs. The 695 nm band is ligand specific, but can be indirectly diminished by gross conformational change. The common description 'conformation sensitive' is misleading.

Schejter and George (1) concluded that the 695 nm band of horse heart ferricytochrome c is conformation sensitive. In contrast, Schechter and Saludjian (2), in their studies with heme undecapeptide, have shown that this band is associated with the binding of a sulphur ligand to the heme iron. Since it is now established (3) that the methionine-80 of cytochrome c is the sixth position ligand of the heme group, the 695 nm band should be present because of this. What then of 'conformation sensitivity'? Quite possibly the protein, in its native conformation, stabilizes the Fe-S bond. If the designation 'conformation sensitive' is apt, then small changes in protein conformation should perturb the Fe-S bond and therefore the 695 nm band. In order to examine this interdependence, we have studied the relationships

of the 695 nm band to the behaviour of the bands around 285, 290, 410, 530, and 655 nm in the presence of conformation perturbants, and CN^-, by difference spectrophotometry (4) at 25°C, over the range 250-850 nm.

The horse heart ferricytochrome c (Type VI, Sigma Chem. Co.) prepared in 0.1 M citrate/phosphate buffer, pH 7.0, was dialysed with excess $K_3Fe(CN)_6$ before use. The guanidine hydrochloride, (Mann Research chemicals) was ultra pure with low absorption in the U.V. at 6M. The difference spectra were recorded on Cary model 14 and 15 spectrophotometers.

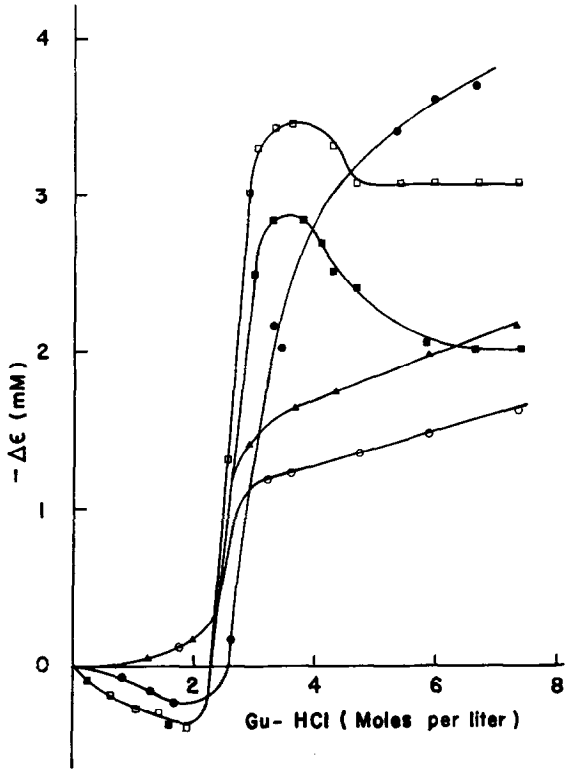

Figure 1 The decrease of extinction coefficient ($-\Delta\varepsilon$) of horse heart ferricytochrome c at 285 (—■—■—), 290 (—□—□—), 530 (—●—●—), 655 (—○—○—), and 695 nm (—▲—▲—) as a function of the concentration of guanidine hydrochloride. Apart from the denaturants, the solution composition was 0.05M NaCl, 0.10M citrate/phosphate buffer, pH 7.0, and cytochrome c: 0.066 mM for 287 and 290 nm, 0.070mM for 655 and 695 nm, and 0.030mM for 530 nm.

Figure 1 shows the changes in the extinction coefficients of the 285, 290, 530, 655 and 695 nm bands brought about by increasing the concentration of guanidine hydrochloride (Gu-HCl). In the 260-300 nm region, the difference spectrum (Fig.2) shows the typical 3 banded pattern that is known to result when the perturbed tryptophanyl and tyrosinyl residues of a protein become less hydrophobic (5). The magnitude of the sharp changes in extinction at 285 and 290 nm is consistent with exposure of TYR-48 and -67 and TRP-59 of cytochrome c to the solvent. The extinction change at 695 nm leads to complete disappearance of the band above about 3.5M Gu-HCl. The behaviour of the Soret band is consistent with the conclusions drawn below, but is too complex to be dealt with here. Table 1 shows the $C_{\frac{1}{2}}$ values for the sudden changes in extinction at the several wavelengths brought about by Gu-HCl and other perturbants. Since the $C_{\frac{1}{2}}$ values for the 695 nm and the protein bands are nearly the same for each perturbant it is clear that 'exposure' of TRP-59 and TYR-48 & -67 only occurs simultaneously with the breaking of the Fe-S bond. This fact

Table I

Values of $C_{\frac{1}{2}}$ for the extinction changes caused by conformation perturbants. ($C_{\frac{1}{2}}$ is the concentration causing half-maximal extinction change. The changes in extinction have been corrected for the solvent effect.)

λ	$C_{\frac{1}{2}}$: Gu-HCl	Urea	Ethylene glycol	KSCN
285 nm	2.55 M	6.35 M	59% (v/v)	3.10 M
290	2.55	6.10	57	3.00
530	3.10	6.60	65	3.10
655	2.60	6.10	61	2.55
695	2.50	6.05	58	2.50

together with the large values of $C_{\frac{1}{2}}$ suggests that 'conformation sensitive' is not an apt description, for the 695 nm band suffers diminution only upon gross distortion of the protein conformation.

To test how the protein conformation depends on the integrity of the Fe-S bond we added 0.1 to 0.5M CN^- to form the cyanide complex. The 695 nm band disappeared as expected, the 530 and 655 bands diminished, and the appearance of the 290 nm region was as given in fig. 2. The absence of the three banded difference spectrum implies a minimal disturbance of TRP and TYR (not more than 2% of the maximal exposure seen in curve B of fig. 2.) We get a similar result with N_3^-. There can be no question that displacing the S-ligand with the CN^-

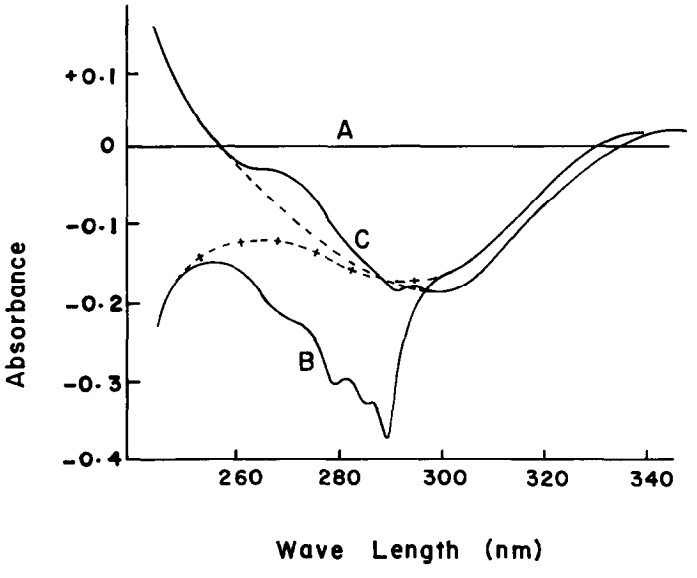

Figure 2. Difference spectra showing the protein bands. A, baseline; B, 0.067mM ferricytochrome c, 0.1M citrate/phosphate buffer pH 7.0, 3.03M Gu-HCl vs no perturbant; C, 0.0384mM ferricytochrome c, same buffer, 0.159M CN^- vs no CN^-. The zero lines +--+-- and ---- for B and C respectively are our estimate of the changes caused by solvent perturbation in 260-300 nm region that cannot be attributed to TRP or TYR. The $\Delta\varepsilon$ values for the UV bands in fig. 1 are based on this estimate.

ligand must produce a small conformation change around the heme, and we see the reflection of this in the spectrum of the 290 nm region.

Thus our experiments show that the 695 nm band of ferricytochrome c disappears when the native MET-80 S-ligand is displaced, and that this can be accomplished directly by ligand competition, with a very small disturbance of the protein conformation as reflected in the 290 nm region, or indirectly by agents which produce a gross change in conformation, sufficient to cause maximal exposure of TRP-59 and TYR-48 and -67 to the solvent. Our conclusion is that the 695 nm band is ligand specific, and conformation insensitive. Continued use of the common description 'conformation sensitive' can only mislead.

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