An Investigation of Protein Conformation of Cytochrome *c* by using Cytochrome *c* Insolubilized on to Agarose Gel

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Cytochrome *c* insolubilized on to agarose gel was shown to be more resistant to denaturation and carboxymethylation than the soluble protein. These differences are discussed both with respect to the conformational changes that take place during denaturation of cytochrome *c* and with respect to the pH-dependent forms of carboxymethyl-cytochrome *c*.

Cytochrome *c* is an electron-transferring haemo-protein in which the single haem moiety is almost completely buried within the protein, the fifth and sixth co-ordination positions of the iron atom being bonded to histidine-18 and methionine-80 (Takano *et al.*, 1972). The iron atom is thus not normally accessible to iron-binding ligands unless conformational changes take place which break or weaken the iron–protein bond or open the haem crevice to allow access. In the oxidized state, phenylalanine-82 does not block the crevice opening (Takano *et al.*, 1972) and this allows cyanide to displace methionine-80 as a weak sixth ligand at pH 7.0 (Takano *et al.*, 1972; Schejter & George, 1965). At extremes of pH, other ligands, including CO and CN[−], will readily act as a sixth ligand (Theorell & Åkesson, 1941; Moore *et al.*, 1975), and when methionine-80 is carboxymethylated the iron atom will also bind O₂ at pH 7.0 (Schejter & Aviram, 1970). Significant protein-conformational changes appear to be implicated in these examples with resultant solvent access to the iron. In a previous paper, Moore & Greenwood (1975) showed that insoluble ferricytochrome *c* has an absorption band at 695nm, and investigated the stability of the iron–methionine-80 bond by comparison of the temperature-dependent changes of this band for soluble and insoluble ferricytochrome *c*. The 695nm band, which has been shown to reflect the integrity of the haem crevice and the iron–methionine-80 linkage (Schejter & George, 1964; Greenwood & Wilson, 1971), is extremely sensitive to denaturants and denaturing conditions, and disappears on reduction of ferricytochrome *c*. Moore & Greenwood (1975) showed that the disappearance of this band with increasing temperature occurred less readily in the insoluble

form, and this was correlated with a smaller standard entropy change. The present paper extends this line of investigation by assessing the effects of denaturants and carboxymethylation on insolubilized cytochrome *c*.

Materials and Methods

Horse heart cytochrome *c* was supplied by Boehringer Chemical Co., London W.5, U.K., agarose powder by Miles-Seravac (Pty.) Ltd., Maidenhead, Berks., U.K., and all other chemicals were AnalaR grade. All solutions were prepared in distilled water. Cytochrome *c* concentrations were determined spectrophotometrically by using a millimolar extinction coefficient of 27.6 for the reduced material at 550nm (Margoliash & Frohwirt, 1959).

Cytochrome *c* was insolubilized on to agarose-gel suspensions by the method of Kay & Lilly (1970). The suspensions were made by blending 1 vol. of agarose gel of the desired concentration with 2 vol. of water, until the suspension was able to pass through a 25-gauge Luer hypodermic-syringe needle. Insoluble and soluble cytochrome *c* were carboxymethylated by the method of Schejter & George (1965).

Absorption spectrophotometry was performed either on a Unicam SP.700A spectrophotometer, or on a split-beam scanning instrument constructed according to the design of Yang & Legallais (1954), by using a Hilger and Watts Monospek 600 monochromator filter, or an Aminco–Chance dual-wavelength/split-beam recording spectrophotometer.

Sodium dithionite and potassium ferricyanide were used to reduce and oxidize cytochrome *c* respectively, and the oxidant or reductant was removed before the experiment by successive washing and centrifugation of the insoluble cytochrome *c*, or by passing soluble

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cytochrome *c* solutions through a Sephadex G-25 column (20cm×2cm).

As discussed in a previous paper (Moore & Greenwood, 1975), the absorbance of insoluble cytochrome *c* at a specified wavelength will have three contributions, i.e. (a) the absorbance due to cytochrome *c*, (b) the absorbance of the gel, and (c) the 'hiding effect' caused by the presence of localized concentrations of chromophores, the middle of which may have 0% transmission, thus effectively masking the chromophore behind it from the light-beam. Agarose gel has very little absorption in the range 400–730nm by virtue of its chemical nature, but over this region scatters light in a wavelength-dependent manner. Anomalous scatter was largely eliminated by using difference spectra, and this had a further advantage, as it minimized any settling effects of the suspension during the scanning time. Only peak locations for the soluble and insoluble species, and not extinction coefficients, could be compared.

Results

Effects of oxidation/reduction and pH on the spectrum of insoluble cytochrome c between 400 and 600 nm

The difference spectrum between sodium dithionite-reduced and potassium ferricyanide-oxidized insoluble cytochrome *c* was indistinguishable from that of the soluble form. Difference spectra between 50%-reduced cytochrome *c* and that where the percentage reduction had been altered by changes in pH showed similar behaviour for soluble and insoluble cytochrome *c*, over the pH range 2–12.

Effect of ionic strength, pH and urea on the 695 nm band of insoluble ferricytochrome c

The 695 nm band of insoluble ferricytochrome *c* is not sensitive to NaCl concentrations at pH 7.0, up to a concentration of 4.5M. This is in contrast with soluble ferricytochrome *c*, where a 10% bleaching of the 695 nm band occurs in the region $I = 0.02$ – 1.0 M. It may be that the chemical processes which lead to the loss of the 695 nm band of soluble ferricytochrome *c* have occurred during insolubilization and resulted in a slightly smaller size of the 695 nm band of insoluble ferricytochrome *c* (Moore & Greenwood, 1975). Alternatively, the lack of sensitivity of the insoluble form to ionic strength may indicate that the covalently bonded gel-protein structure does not allow a loosening of the general protein conformation and consequently stabilizes the iron-methionine-80 linkage.

The effect of pH on the 695 nm absorbance of soluble and insoluble ferricytochrome *c* at room temperature is shown in Fig. 1. The titration profiles for soluble ferricytochrome *c* were shown by Greenwood & Wilson (1971) to give pK values of 3.18 ± 0.03 and 8.9 ± 0.06 in 0.2M-KCl, and 9.0 and 3.1 in 0.02M-KCl, as measured from the points of maximum slope. The titration profiles for insoluble ferricytochrome *c* at both ionic strengths are identical, and give pK values of 3.55 ± 0.10 and 9.25 ± 0.10 . Both the latter values show a shift towards alkaline pH, which may indicate that the pH at the surfaces of the gel matrix is more acidic than that in the solution as a whole. It is possible that the acidic groups responsible are the residual sulphate groups in the agarose. The lack of

Fig. 1. Effect of pH on the 695 nm band of insoluble ferricytochrome *c*

The concentration of the supporting gel was 2% (w/v), and the initial concentration of insoluble ferricytochrome *c* was 0.56 mg/ml of suspension. The absorbances at 695 nm in 0.2M-KCl solution (○) and in 0.02M-KCl solution (●) are shown (a). (b) The plot of the pH titration for soluble ferricytochrome *c*, which is identical with that of Greenwood & Wilson (1971). The continuous line plots the titration in 0.2M-KCl solution and the broken line in 0.02M-KCl solution. The experiments were performed by back-titrating soluble or insoluble ferricytochrome *c* in NaOH or HCl to pH 7.0 with HCl or NaOH solutions respectively. The suspensions or solutions were made up in water before HCl or NaOH was added; all absorbance readings were taken in a 1 cm spectrophotometer cell.

Fig. 2. (a) Effect of urea on the 695 nm band of insoluble ferricytochrome *c*, and (b) spectra of insoluble ferricytochrome *c* in the region 660–730 nm

The concentration of the supporting gel was 2% (w/v). All suspensions were made up in 0.02 M-sodium phosphate buffer, pH 7.0, and all readings were taken in 1 cm spectrophotometer cells. [Cytochrome *c*] = 0.54 mg/ml of suspension. (a) With varying [urea] and (b) with no urea present (—) and with urea to saturation (---).

effect of KCl concentration on the profiles for insoluble ferricytochrome *c* is consistent with the lack of effect of ionic strength on the 695 nm band at pH 7.0.

The 695 nm band of insoluble ferricytochrome *c* was bleached by urea with a midpoint of approx. 3.0 M-urea, which is far less than the equivalent value of 6.5 M found by Stellwagen (1968) for soluble ferricytochrome *c*. At the end of the titration some residual absorbance at 695 nm remained as a shoulder on the spectrum, which was not removed by addition of solid urea up to saturation. The results are presented in Fig. 2.

Carboxymethyl-cytochrome *c*

Carboxymethylation of ferricytochrome *c* results in a molecule in which methionine residues 65 and 80 are modified to the carboxymethyl derivatives. In this state, modified methionine-80 can no longer act as a sixth ligand to the iron atom, and the modified cytochrome *c* has an altered absorption spectrum and binds ligands in conditions where the native molecule will not. These new properties have been entirely attributed to the modification of methionine-80 (Schejter & Aviram, 1970).

Preparation of the carboxymethyl forms of soluble and insoluble ferricytochrome *c* were performed as described by Schejter & Aviram (1970), and at intervals during preparation 0.1 ml samples were

taken from the reaction medium and diluted to 20 ml with 0.1 M-sodium phosphate buffer, pH 7.0. The absorption of the sodium dithionite-reduced material at 414 nm was recorded in the presence (E_{co}) and absence (E_r) of saturating amounts of CO. The ratio, E_{co}/E_r , is a measure of the reaction, as only carboxymethyl-ferricytochrome *c* will bind CO at pH 7.0. The progress curve of this reaction indicates simple monophasic kinetics, the second-order rate constant being $0.33 \text{ M}^{-1} \cdot \text{h}^{-1}$ at room temperature (approx. 18°C), which is slower than that calculated for soluble cytochrome *c* by Dr. M. Wilson (personal communication) of $1.00 \text{ M}^{-1} \cdot \text{h}^{-1}$ under the same conditions.

The spectrum of the insoluble carboxymethyl-ferricytochrome *c* at pH 7.4 was identical with that of the soluble form discussed by Schejter & George (1965) (with respect to peak positions), having peaks at 550 and 520 nm. These bands intensified on raising the pH to 10.0 and weakened on lowering the pH to 6.0 (Schejter & George, 1965), the two bands becoming a single band with a peak at 550 nm at pH 4.0.

Schejter & George (1965) found that the spectrum of soluble carboxymethyl-ferricytochrome *c* at pH 4.0 showed a broad peak with a maximum at 490 nm, a small band at 622 nm, and an inflexion at 550 nm. At pH 6.0 these bands were replaced by a single band at 528 nm which did not change when the pH was increased to 10.0. Insolubilized carboxymethylated

ferricytochrome *c* was found to have a single broad band with a maximum at 528 nm which showed progressive intensification as the pH was increased from 4.0 to 10.0.

Discussion

The spectral similarities found for soluble and insoluble ferri- and ferro-cytochrome *c* and the presence of the 695 nm band in the spectrum of the ferri- form suggests that insolubilization has not fundamentally changed the molecule. However, once insolubilized cytochrome *c* is protected to a varying degree from protein-denaturing processes which influence soluble cytochrome *c*. Changes that do occur are of a similar type, as shown by the influence of pH on the 695 nm band, where the *pK* values measured for insoluble ferricytochrome *c* almost certainly arise from the same ionizations as those of the soluble form.

Lambeth *et al.* (1973) suggest that the *pK* in the region of pH 9.0 arises when lysine-79 is co-ordinated to the iron atom as the sixth ligand instead of methionine-80. In view of the spectral similarities between soluble and insoluble cytochrome *c* it appears that not only is methionine-80 the sixth ligand at neutral pH in insoluble ferricytochrome *c* (i.e. there is a 695 nm band) but that it is displaced by lysine-79 at high pH values.

The denaturation of soluble ferricytochrome *c* has been studied by several workers, and three separate changes, with midpoints of approx. 3.0, 6.5 and 7.1 M-urea have been identified (Stellwagen, 1968; Myer, 1968; Kaminsky *et al.*, 1972). Stellwagen (1968) has interpreted the three transitions as first, some displacement of the polypeptide chain from the haem moiety, exposing the haem to solvents, followed by disruption of the protein-haem covalent bond(s), allowing limited unfolding of the polypeptide chain, and lastly, gross unfolding of the polypeptide chain. The change with a midpoint of 3.0 M-urea and the residual 695 nm shoulder found in experiments with insoluble ferricytochrome *c* indicate that the urea-dependent 695 nm absorbance change of soluble ferricytochrome *c* is a function of two mutually aided processes. That is, small polypeptide-chain displacements lead to rupture of the sixth ligand co-ordination to the iron atom, which in turn leads to an opening of the crevice structure and an increase in polypeptide-chain displacements. Limiting or preventing the second process should result in only a limited loss of the 695 nm band. This is consistent with the results of Kaminsky *et al.* (1972), who observed that urea denaturation at pH 7.0 proceeded in two stages as measured by the ability of ferricytochrome *c* to bind CO, the midpoint for the first stage being 3.6 M-urea, and that for the second and major change at 6.5 M-

urea. When the pH was lowered to 5.0, Kaminsky *et al.* (1972) found that the amount of CO bound was considerably increased during the first process at the expense of the second, without changing the mid-points.

The carboxymethylation of cytochrome *c* is thought to proceed by initial binding of cyanide to the iron atom, displacing methionine-80 as the sixth ligand and opening the crevice. The final value of E_{co}/E_r for insoluble carboxymethyl-cytochrome *c* is 1.35, in comparison with 1.95 for the soluble form. Part of this discrepancy may be due to the properties of the chromophore/agarose-gel system. First, there is a small value for the absorbance of the gel added to both absorption components of the ratio, and secondly there may be a contribution by the 'hiding effect'. An evaluation of the magnitude of both can be undertaken assuming that the absorption of the gel remains constant at 414 nm during the reaction. If the absorbance of the gel were fully responsible for the difference, its value would have to be 1.72 times the absorbance of the reduced cytochrome *c* at 414 nm, and this is clearly unacceptable, and the 'hiding effect' cannot play a major role, as linear absorbance changes at 414 nm are observed during CO titrations of insoluble carboxymethyl-cytochrome *c* at pH 7.0 (Moore & Greenwood, 1975). Clearly insolubilization must affect the spectrum of cytochrome *c*, and/or carboxymethyl-cytochrome *c*, and/or the CO derivatives.

The effect of pH on the spectrum of soluble carboxymethyl-cytochrome *c* has been extensively studied by Schejter & George (1965) and Schejter & Aviram (1970), and they suggest that the spectrum of soluble carboxymethyl-ferricytochrome *c* at pH 4.0, which shows characteristics of high-spin ferri-haemoprotein derivatives, implies that the iron atom is co-ordinated to at least one weak-field ligand. Schejter & Aviram (1970) suggest that the single haem-linked ionization in the pH range 4.0–6.0 was due to an electronic or conformational isomerism between purely high-spin and purely low-spin molecules, having different residues co-ordinated to the iron atom. Extrapolation of these ideas to the results for insoluble carboxymethyl-ferricytochrome *c*, where no such high-spin spectrum is seen, indicates that a certain change in conformation is required in order to acquire a new spectrum. This is consistent with the slow rate of formation of the new spectral type observed by Schejter & Aviram (1970) when acidifying soluble carboxymethyl-ferricytochrome *c* from neutrality to pH 4.0.

In the same way that soluble chemically modified forms of cytochrome *c* have shed light on the role of many of the amino acids of the cytochrome *c* molecule, insolubilization can be used to indicate the importance of conformation integrity, and this is particularly valuable for cytochrome *c*, as the con-

formational integrity of the molecule appears to have been modified very little during insolubilization.

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