The 695-mμ Band of Ferricytochrome c and Its Relationship to Protein Conformation*

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The 695-mμ band of ferricytochrome c at neutral *p*H can be abolished by increasing the temperature and by adding denaturing agents or iron-binding ligands. These spectroscopic modifications are fast and reversible. Between 0.07 and 1.7 mM concentrations Beer's law is obeyed by the 695-mμ band of ferricytochrome c, intimating that changes in aggregation are not involved in the transformations that cause the disappearance of the band. Addition of each of several nitrogenous bases to heme and hemoprotein solutions devoid of a 695-mμ band did not cause the appearance of this band, suggesting that the 695-mμ band is determined by structural features other than the chemical nature of the iron ligands. This result and the effect of denaturing agents are interpreted as indications that the 695-mμ band is intimately related to the overall conformation of the protein moiety of ferricytochrome c. The effect of temperature on the band can be considered as a conformational isomerism between a "cold" and a "hot" form of the hemoprotein, stable at low and high temperatures, respectively. At 25° this equilibrium is characterized by the following thermodynamic parameters: $\Delta F = +1.7$ kcal/mole; $\Delta H = +14.6$ kcal/mole; $\Delta S = +43$ eu. These values are of an order of magnitude compatible with changes in protein conformation. At 38° about 15% of the molecules are in the "hot" conformation, implying a possible role of this isomerism in biochemical processes in which cytochrome c participates.

In its oxidized state, mammalian cytochrome c has an absorption band in the red region of the spectrum, characterized by a maximum at 695 mμ and an inflection at 655 mμ. This band was first described by Theorell and Akesson (1939), who also showed that it disappears at *p*H lower than 2.5 and higher than 9.35 (Theorell and Akesson, 1941).

It was demonstrated later that the band was abolished when the cyanide or azide complexes of ferricytochrome c are formed (Horecker and Kornberg, 1946; Horecker and Stannard, 1948). More recent investigations have shown that the band is very sensitive to temperature changes (Schejter *et al.*, 1962) and that it is absent in the polymeric forms of ferricytochrome c (Schejter *et al.*, 1963).

A study of the factors which affect the 695-mμ band of monomeric mammalian ferricytochrome c in aqueous solutions is presented in this paper.

Materials and Methods

Most of the experiments were performed with cytochrome c extracted from beef hearts (Keilin and Hartree, 1945) and purified chromatographically on columns of Amberlite CG-50 (Margoliash, 1957). Only the fraction eluted with 0.25 M NaCl was used; in this fraction the cytochrome c molecule is present as a monomer (Margoliash and Lustgarten, 1962). Some experiments were carried out with horse heart cytochrome c (Sigma Chemical Co., Type III) purified by the same procedure. A sample of horse heart cytochrome c dimer, containing 9.3% monomer (Margoliash and Lustgarten, 1962), was a gift of Dr. E. Margoliash. The pepsin "core" or hemopeptide of cytochrome c was prepared as indicated by Margoliash *et al.* (1959).

All other chemicals used were commercial samples of analytical grade.

Spectrophotometric observations at fixed wavelengths were made in a Beckman DU spectrophotometer; complete spectra were recorded in a Beckman DK-1 spectrophotometer. In both instruments, the temperature of the cell compartments was kept constant by water circulation. The *p*H values were measured with a glass electrode using a Beckman Model GS *p*H meter.

Results

Effect of Temperature on the 695-mμ Band.—Figure 1 shows the effect of temperature on the optical density at 695 mμ of beef and horse ferricytochrome c. In this experiment the temperature of a 6.5×10^{-4} M solution of ferricytochrome c in sodium phosphate buffer at *p*H 7.0 and ionic strength 0.01 was gradually increased from 2 to 60°. The intervals between consecutive readings, including the time necessary for temperature change and equilibration, were of the order of 10 minutes. The smooth decrease of the optical density observed between 10 and 60° was interrupted by a rapid increase owing to the formation of turbidity in the solution. Such precipitations always occurred when cytochrome c was heated at this concentration, but they were not observed when the hemoprotein was heated in more dilute solutions, of the order of 10^{-6} M.

If the heating of the cytochrome c solutions was halted before reaching the point of appearance of turbidity, and the solutions were cooled again, the optical changes observed were reversed and the entire band reappeared. The cycle of heating and cooling the cytochrome c solutions could thus be repeated many times, and the effect on the 695-mμ band was always the same, indicating the reversibility of the changes observed. On the other hand, if the solutions were kept for periods of several hours at temperatures between 40 and 50°, upon cooling the height of the 695-mμ band did not reach its original value, the optical density at 10° being slightly less than the initial value before heating. These irreversible changes are extremely slow: in order to observe a 10% decrease in the height of the band the solutions must be kept at 45° for 48 hours.

An attempt was made to study the rate of the reversible transformation in the following manner. A solution of the hemoprotein at 10° was rapidly brought to 70° by immersing the cuvet in hot water and placing it in the cell compartment thermostated at the same temperature. The decrease of the optical density at

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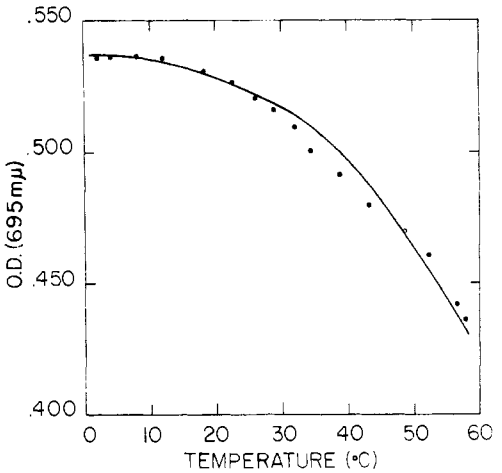

FIG. 1.—Effect of temperature on the optical density of 6.5×10^{-6} M ferricytochrome c in phosphate buffer at *p*H 7.0, ionic strength 0.01. The points indicate actual experimental readings; the full line was calculated by assuming a configuration isomerism between two forms of the hemoprotein, stable at low and high temperatures, respectively (see Discussion).

695 mμ was immediately observed, and no further significant change was apparent during the short period remaining until the onset of turbidity. Similarly, if a rapidly heated solution was immersed in a refrigerating bath at 0° and immediately placed in a cooled cell compartment, the optical density was that characteristic of cold ferricytochrome c. These experiments show that the reversible changes of the 695-mμ band are very fast, since no more than 45 seconds elapsed between the immersion of the cuvet and the spectrophotometric reading.

The spectra of ferricytochrome c at 10 and 45°, between 650 and 750 mμ, are shown in Figure 2. If these spectra are compared to those of monomeric and dimeric ferricytochrome c recorded at 11° (Schejter *et al.*, 1963), it can be seen that upon heating the spectrum of the monomer tends toward that of the cold dimer.

Effect of Dilution on the 695-mμ Band.—The parallelism between the effects of heating and dimerization on the spectroscopic properties of ferricytochrome c suggested, as a possible explanation for the temperature dependence of the 695-mμ band, that of a reversible change of aggregation of the hemoprotein. If this were the case, deviations from Beer's law might be expected in the spectral region concerned under the conditions of concentration and temperature of these experiments.

In order to test this possibility, the optical densities of ferricytochrome c solutions at 695 mμ for concentrations ranging from 0.07 to 1.74 mM were measured at 10 and 45°. From these values, extinction coefficients were calculated and plotted against the logarithms of the concentrations at which they were measured. The result is shown in Figure 3, which indicates that at 695 mμ ferricytochrome c does however obey Beer's law.

Effect of Denaturing Agents on the 695-mμ Band.—When the spectra of 0.3 mM solutions of ferricytochrome c at *p*H 7.0 in 4 M urea, 15% ethanol, 0.1 M trichloroacetate, or 6 M guanidine were recorded between 600 and 750 mμ, varying degrees of depression of the 695-mμ band could be observed. The optical densities at 695 mμ measured under these conditions are listed in Table I.

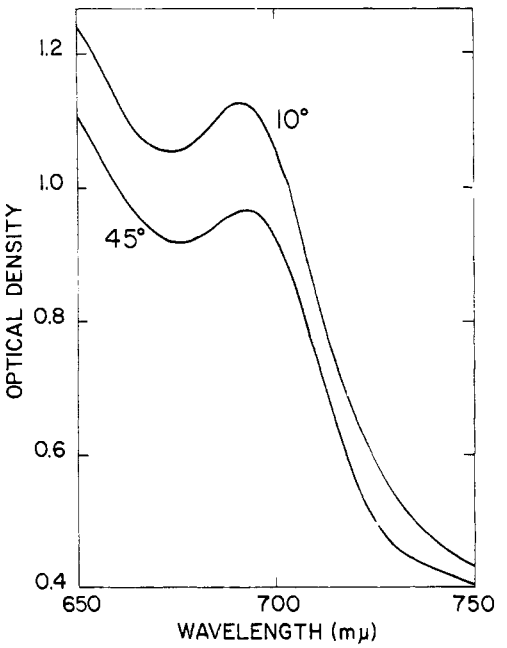

FIG. 2.—The spectrum of neutral ferricytochrome c at 10° and 45°, in the 695-mμ band region.

TABLE I

EFFECT OF DENATURING AGENTS ON THE OPTICAL DENSITY OF FERRICYTOCHROME C AT 695 mμ

Denaturing Agent	Optical Density[a]
None	0.235
4 M Urea	0.195
15% Ethanol	0.205
0.1 M Trichloroacetic acid	0.125
0.1 M Trichloroacetic acid after 24-hr dialysis	0.200
6 M Guanidine	0.112
6 M Guanidine after 48-hr dialysis	0.215

[a] The optical densities were measured on 0.3 mM solutions of monomeric ferricytochrome c in 0.1 M phosphate buffer, at *p*H 7.0 and 11°. In two cases the measurements were repeated after prolonged dialysis of the solutions against buffer.

The reversibility of the changes induced by these denaturing agents was also tested. The solutions were dialyzed against buffer for 48 hours and their spectra were recorded again; it was observed that the entire band reappeared in all cases. The optical densities measured after the dialysis at 695 mμ are also listed in Table I. In Figure 4 the entire band is shown in the presence of 6 M guanidine, and after dialysis; in Figure 5 the optical densities of ferricytochrome c solutions at 695 mμ are plotted against the various concentrations of guanidine at which they were measured.

Attempts to Create the 695-mμ Band.—Experiments were carried out to see whether the 695-mμ band could be created by the deliberate addition of nitrogenous based to heme and hemoprotein solutions for which it is absent. Imidazole, histidine, butylamine, and glycine in concentrations of up to 2 M were added to ferric hemin, to the ferric form of the pepsin hemopeptide of cytochrome c, to ferrimyoglobin and ferrihemoglobin, and to dimeric ferricytochrome c. These additions were made at 10° in buffered solutions at *p*H 7.0 and 10.0, in phosphate and bicarbonate buffers, respectively, and the spectra were recorded. The results were always negative: absorption bands were

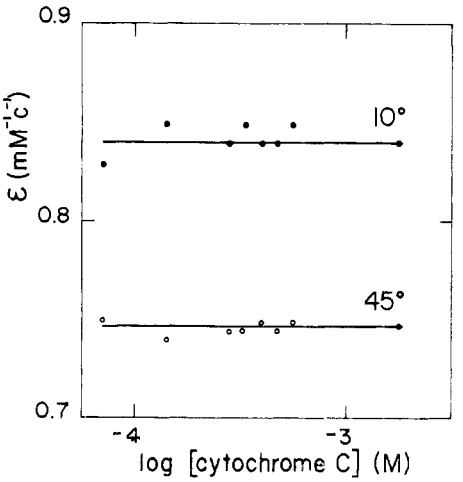

FIG. 3.—Millimolar extinction coefficients of ferricytochrome c versus concentration, at two different temperatures. Absorbance measured in 0.1 M phosphate buffer at 695 mμ, *p*H 7.0.

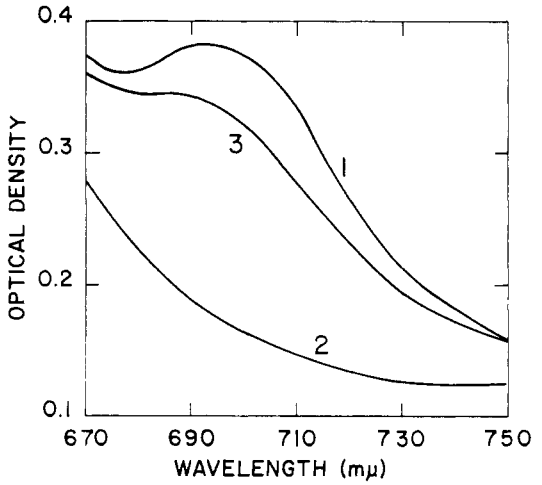

FIG. 4.—Effect of reversible denaturation on the 695-mμ band of ferricytochrome c. Spectra of ferricytochrome c (curve 1); ferricytochrome c in 6 M guanidine (curve 2); ferricytochrome c in 6 M guanidine, then dialyzed for 48 hours against phosphate buffer (curve 3). Spectra recorded at 10°, *p*H 7.0.

not observed in the red region, between 600 and 750 mμ.

However an unexpected result of these experiments was that imidazole added to the cytochrome c dimer sample which contained 9.3% of monomer abolished the small shoulder at 695 mμ due to the monomer (Schejter *et al.*, 1963). This suggested a reaction between imidazole and monomeric ferricytochrome c, which was confirmed by the further observation that addition of imidazole to the pure monomeric hemoprotein resulted in the complete disappearance of the 695-mμ band. The visible and Soret regions of the spectrum of the monomer did not change upon addition of imidazole. In an additional experiment a solution of 0.5 mM monomeric ferricytochrome c in 0.1 M phosphate buffer, *p*H 7.0, contained in a spectrophotometric cuvet, was titrated with imidazole. The solution of imidazole was made in the same buffer, and titrated to *p*H 7.0 with dilute HCl. The titrant was added to the ferricytochrome c with a micropipet, and the extent of the reaction was followed by measuring the optical densities at 695 mμ. The results of a typical experiment are shown in Figure 5; the concentrations of unprotonated imidazole were estimated by assuming a $pK' = 7.0$ for the first ionization of this compound.

DISCUSSION

Possible Origins of the 695-mμ Band of Ferricytochrome c.—In hemoprotein studies advantage is usually taken of the fact that substitution of the ligands coordinated to the heme iron results in spectral changes. It is often assumed that the converse is also true, namely, that spectroscopically detectable changes produced by a variety of treatments are caused by substitution of one of the iron-linked ligands.

There are, however, certain instances in which spectral change does not appear to be synonymous with iron-ligand substitution, but with modification of the conformation of the heme-linked polypeptide chain. A typical case is that of the polylysine complexes of hemin (Blauer, 1961). Our observations on ferricytochrome c tend to show that its absorption band with maximum at 695 mμ is intimately related to the overall conformation of the protein moiety.

In the first place, the reversible changes of the band caused by urea, guanidine, trichloroacetate, and ethanol can hardly be attributed to iron binding by these reagents. If trichloroacetate or ethanol were replacing an imidazole group coordinated to the iron, drastic changes in the rest of the visible and Soret spectra would surely occur, but none were observed. Furthermore, the total change observed with guanidine occurs between 2 and 4 M concentrations of this compound, while the stoichiometry of the reactions of cytochrome c with ligands requires 100-fold span in the concentrations of the latter for the completion of the observed change. It seems far more reasonable to explain the effect of denaturing agents on ferricytochrome c as being exerted upon the secondary and tertiary structures of the protein, changes which are reflected by the modification observed in the red region of the spectrum.

This interpretation is also consistent with the negative results of the experiments designed to create the band in other heme and hemoprotein solutions for which it is absent. In these experiments, the primary structure of the iron-coordination octohedron of cytochrome c, constituted by four pyrrole nitrogens and two imidazole nitrogens (Theorell, 1941; Ehrenberg and Theorell, 1955) or one imidazole and one ε-amino nitrogen (Margoliash *et al.*, 1959), was obtained by addition of the appropriate ligands to hemin, to the cytochrome c hemopeptide, to myoglobin, and to hemoglobin. None of the complexes formed showed the characteristic absorption in the red region of the spectrum. Therefore structural parameters other than the chemical nature of the iron ligands must be involved in the transition responsible for the 695-mμ band.

The effect of imidazole on the 695-mμ band provides further corroboration for this interpretation. The shape of the titration curve determined spectrophotometrically at 695 mμ (Fig. 5) is entirely consistent with the stoichiometry of a ligand-substitution reaction; and the existence of imidazole complexes of hemoproteins and hemopeptides is well known (Scheler *et al.*, 1957; Harbury and Loach, 1960a,b). Hence the effect of imidazole can be attributed to the formation of a ferricytochrome c–imidazole complex. In this complex the iron ligands are the same as in free ferricytochrome c, since the protein group displaced from iron coordination when ferricytochrome c complexes are formed is a histidine imidazole (George *et al.*, 1963). However, while the rest of the visible

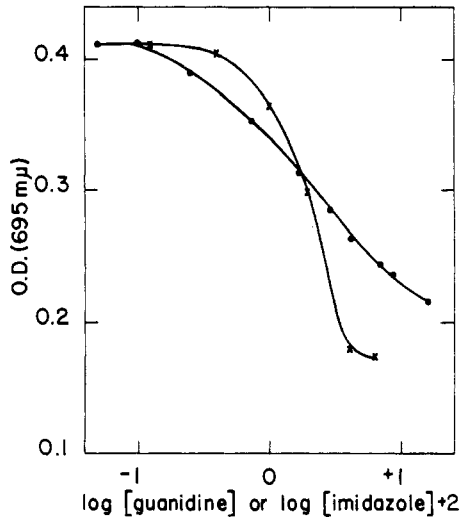

FIG. 5.—Spectrophotometric titrations of 0.5 mM ferricytochrome c with guanidine (×—×) and with imidazole (●—●). Titrations performed in 0.1 M phosphate buffer, *p*H 7.0, at 11°.

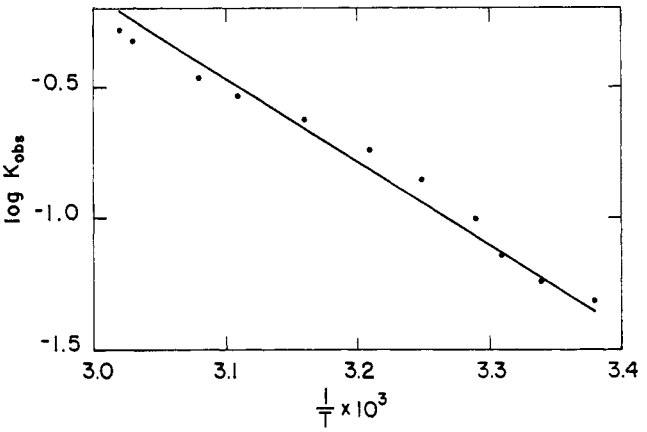

FIG. 6.—A plot of log K_{obs} versus $1/T$ for the reaction: ferricytochrome c "cold" ⟷ ferricytochrome c "hot." Values of K_{obs} estimated from the experimental data of Fig. 1 by using the equations given in the text.

spectrum of ferricytochrome c–imidazole is identical to that of the free hemoprotein, the 695-mμ band of the latter is absent in the former. Thus we see again that the nature of the ligands coordinated to the iron atom of ferricytochrome c is not sufficient to account for the origin of the 695-mμ band.

On the basis of the preceding considerations, we feel justified in attributing the origin of the 695-mμ band to some feature in the conformation of the protein and its relationship to the heme group. Several possibilities may be considered.

First, the transition may arise from the interaction of the heme group with a specific configuration of the protein moiety. A relationship between peptide conformation and the spectrum of a hemopolypeptide has been established by Blauer (1961).

In the second place, the transition may be due to a specific orientation of the plane of the iron-linked imidazole of ferricytochrome c with respect to the heme group; the way in which this structural feature may affect the heme-linked physical properties of the cytochrome c molecule has been discussed in detail by Lumry and co-workers (Lumry, 1961; Lumry *et al.*, 1962).

Finally, recent information resulting from X-ray diffraction studies on metalloporphyrins (Fleischer, 1963a,b) and hemoproteins (Kendrew, 1963) shows that the tetrapyrrolic ring is not exactly planar. At least in the crystalline state, the geometry of the porphyrin ring can be temperature dependent, even at room temperatures (Hoard *et al.*, 1963), and a similar flexibility could be expected with even more reason in the case of protein-bound porphyrins in solution. Hence it is also possible that the heme group of cytochrome c has a peculiar conformation of its own responsible for the 695-mμ band.

Conformation Isomerism.—Perhaps the most striking feature of the 695-mμ band is its disappearance when the temperature is raised. The fact that it occurs over a wide range of temperature, together with its reversibility, precludes the possibility of a higher-order phase transition, such as an internal melting.

Two types of chemical reaction can be considered to explain the phenomena: a change in aggregation, or a change in protein conformation.

The possibility of a change in aggregation merits special consideration because in the polymeric forms of ferricytochrome c which are formed irreversibly the 695-mμ band is absent (Schejter *et al.*, 1963). However, the finding that Beer's law is obeyed at the concentration and temperature of the experiments reported here speaks against a polymerization of the monomeric cytochrome c molecule under these conditions, although it does not rule it out completely.

Moreover, it has been demonstrated by Margoliash and Lustgarten (1962) that 6 M guanidine at neutral *p*H readily depolymerizes the otherwise stable aggregated forms of cytochrome c; and we have shown above that 6 M guanidine reversibly abolishes the band in the monomer. This demonstrates that a form of monomer exists which does not show the band even at low temperatures.

In view of the similarity between the spectroscopic changes observed when monomeric ferricytochrome c is heated, and when it is treated with 6 M guanidine at low temperatures, we interpret the temperature effect as the displacement of an equilibrium between two conformational isomers of the monomeric hemoprotein, a "cold" form in which the 695-mμ band is present, and a "hot" form in which the band is absent:

"cold" ferricytochrome c — "hot" ferricytochrome

For this reaction, the observed equilibrium constant, K_{obs}, can be measured spectrophotometrically:

$$K_{obs} = \frac{(OD)_t - (OD)_c}{(OD)_h - (OD)_t}$$

where $(OD)_t$ is the optical density at any temperature, and $(OD)_c$ and $(OD)_h$ are the optical densities of the "cold" and "hot" forms, respectively. The optical density of the "cold" form is readily available because the equilibrium is entirely displaced in its favor below 10°. The optical density of the "hot" form cannot be found experimentally because the reaction does not reach completion before precipitation sets in at about 60°. It is possible, however, to get an approximate value because of the similar extinctions of all types of ferricytochrome c that lack the 695-mμ band. Thus, one can assume for the "hot" form an extinction coefficient $\epsilon_{mM} = 0.38$, the value obtained from the corrected spectrum of the dimer (Schejter *et al.*, 1963), or alternatively, $\epsilon_{mM} = 0.40$, the value calculated for ferricytochrome c in 6 M guanidine or in 0.1 M trichloroacetate (Table I). Since the concentration of ferricytochrome c in any experiment is known, the optical density corresponding to the "hot" form can be estimated.

Adopting the value $\epsilon_{mM} = 0.38$, we have calculated the values of K_{obs} from the data shown in Figure 1 for a series of temperatures and plotted their logarithms against $1/T$ (Fig. 6). The plot is satisfactorily linear, and by the least squares method the observed enthalpy change can be estimated as $+14.6 \pm 0.1$ kcal/mole.

At 26°, $K_{obs} = 0.059 \pm 0.003$, hence the free-energy change at this temperature is $+1.73 \pm 0.03$ kcal/mole, and the entropy change at 26° is calculated as 43.2 ± 0.4 eu.

These thermodynamic parameters show that the conversion of the "cold" form of ferricytochrome c into the "hot" form is attended by a very unfavorable enthalpy change, and a markedly favorable entropy change. Such values are characteristic of the reversible denaturation of proteins, and provide additional evidence in favor of the structural interpretation given above.

There are also other phenomena that can be explained by the conformation isomerism of the ferricytochrome c molecule suggested here. Irreversible polymers of cytochrome c are formed when solutions of the enzyme are subjected to the action of trichloroacetate (Nozaki, 1960; Margoliash and Lustgarten, 1962). But it has been shown in this study that there is initially a reversible effect of trichloroacetate which can best be explained by a conformational change in the monomeric protein. We therefore suggest that polymerization occurs only when the molecule is in its "hot" or reversibly denatured conformation.

$$n \text{ "hot" ferricytochrome c} \rightleftharpoons (\text{"hot" ferricytochrome c})_n$$

The absence of the 695-mμ band in the oxidized polymeric forms follows naturally, because the monomeric units present in the polymer are "frozen" in the "hot" configuration, for which the 695-mμ band is already absent.

The disappearance of the 695-mμ band when complex formation occurs with cyanide and azide (Horecker and Kornberg, 1946; Horecker and Stannard, 1948), as well as the effect of imidazole described here, can also be explained in terms of conformation changes. Since ferricytochrome c has a "closed-crevice" structure (George and Lyster, 1958), complex formation requires the breakage of one of the iron-protein bonds, a process accompanied by an entropy increase of about 60 eu (George and Tsou, 1952). This change has been related to conformation changes in the protein, made possible by the liberation of a portion of its chain from the constraints imposed by the iron-protein linkage (George and Hanania, 1955). Hence it is reasonable to suppose that the structure responsible for the 695-mμ band would again be disrupted; and the similarity between the entropy changes caused by cyanide binding and by heating may be a reflection of similar structural changes involved in both processes.

The absence of the 695-mμ band with ferrocytochrome c does not imply that an analogous isomeric equilibrium is absent in the reduced form. In fact, Okunuki (1961) has observed small changes in its visible spectrum on heating which are reversed on cooling.

From the physicochemical point of view, it is obvious that in the thermodynamic and kinetic analysis of any reaction in which cytochrome c participates conformation changes that occur in either oxidation state must be taken into account. Moreover, the data for ferricytochrome c show that at neutral *p*H and 38°, 85% of the enzyme is present in the "cold" form and 15% in the "hot" form. Hence, if the suggested isomerism occurs *in vivo*, the biological reactions of cytochrome c will certainly reflect it. On the other hand, if cytochrome c in its intracellular environment does not involve the two forms, the thermodynamic parameters for cytochrome c reactions measured in aqueous solutions cannot be applied without qualifications to the analysis of its reactions in its natural environment.

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