The Reaction of Cytochrome *c* with Imidazole*

Abel Schejter and Irit Aviram

ABSTRACT: Ferricytochrome *c* binds imidazole and 1-methylimidazole in reactions with 1:1 stoichiometry. The pH and temperature dependence of imidazole binding indicate that the reaction is analogous to the binding of the enzyme by ligands such as cyanide and azide. At pH 7.4 and 18° the observed thermodynamic parameters for imidazole binding are: $\Delta F = -1.9$ kcal/mole, $\Delta H = +1.1$ kcal/mole, and $\Delta S = +10.2$ eu. The spectral changes induced by imidazole binding are similar to those that occur when cytochrome *c* is dimerized, heated to 50°, or denatured with urea. This result is interpreted as showing that in all the cases mentioned, the iron binding sulfur of methionine-80 is displaced by nitrogenous ligands. At low concentrations, imidazole does not affect the kinetics of the reduction of ferricytochrome *c* by ascorbate, but higher concentrations of the ligand cause a reduction in the rate, while guanidine hydrochloride increases the rate.

The reduction of ferricytochrome *c* imidazole is very fast; the spectrum of the reduced complex is identical with that of the reduced free enzyme, except for an increase of 2% in the absorbancy at the peak of the Soret region. This difference disappears with first-order kinetics, with $k = 1.08 \times 10^{-1}$ sec^{-1} at 21° and an energy of activation of 21.8 kcal/mole.

It was shown by Schejter and George (1964) that the 695-mμ band of ferric cytochrome *c* exists only if the protein is in its native conformation, and that low concentrations of imidazole cause the disappearance of the band. This fact was attributed to the formation of a complex with imidazole as a ligand of the iron atom of cytochrome *c*. Greenwood and Palmer (1965) measured the rate of the reaction between imidazole and cytochrome *c* and found it to be practically independent of the concentration of imidazole; this led them to suggest that the effect of imidazole was due not to a specific iron ligand binding, but to a nonspecific denaturation.

The titrations presented here indicate that the reaction involves binding at a definite site. The effects of pH and temperature on the titration curves are those expected for binding of the cytochrome *c* iron with imidazole, and so are the spectral changes induced by the ligand in the visible region. Reduction of ferricytochrome *c*–imidazole by dithionite is very fast, and the resulting spectrum differs from that of native cytochrome *c* only at the peak of the Soret band; this difference disappears rapidly, in a reaction with first-order kinetics.

Materials and Methods

Horse cytochrome *c* (Sigma Chemical Co., type II) was purified by chromatography on Amberlite IRC-50 (Margoliash and Walasek, 1967). All the materials employed were commercial products of the best degree of purity available. A Cary Model 15 spectrophotometer was employed to record spectra and to measure the kinetics of ferrocytochrome *c*–imidazole decomposition. The kinetic measurements of the reduction by ascorbate, at 550 mμ, were carried on in a Zeiss PMQ II spectrophotometer. pH was measured with a glass electrode in a Radiometer PHM4 instrument. The titrations were performed in the following way: to 2-ml solutions of 0.6 mM ferricytochrome *c* in 0.1 M phosphate buffer, at various pH values, aliquots of concentrated imidazole solutions at the same pH were added with a micropipet. The entire spectra were recorded and the readings at 695 mμ were corrected for dilution. Since the concentration of added ligand exceeded by at least two orders of magnitude that of the protein, the concentration of free imidazole at equilibrium was assumed to equal its total concentration for the calculation of K_{obsd}.

Reduction of ferricytochrome *c* with ascorbate was performed on solutions of the enzyme in 0.05 M phosphate buffer (pH 7.4). The reaction mixtures contained also NaCl in such concentrations that the final ionic strength, resulting from the contribution of the salt and the imidazole, was constant for a given series of experiments. At zero time ascorbate was added, and the change in absorbance at 550 mμ was recorded. The kinetics of decomposition of ferrocytochrome *c*–imidazole were measured at 416 mμ. The solutions contained ferricytochrome *c* (1.2×10^{-2} mM) and imidazole (1.0 M), neutralized to pH 7.0 with HCl. The absorbancies were read against water plus a 1.525 absorbancy filter, using a 0–0.1 absorbancy slide wire. The instrument balance was set arbitrarily. Solid dithionite was added with rapid stirring and a stopwatch was simultaneously started. Between the beginning of the reaction and that of the recording there was an interval of 5 ± 1 sec. Under these conditions, an absorbancy change of 0.030 was observed between the beginning of the recording and the end of the reaction.

* From the Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel. *Received June 5, 1968.*

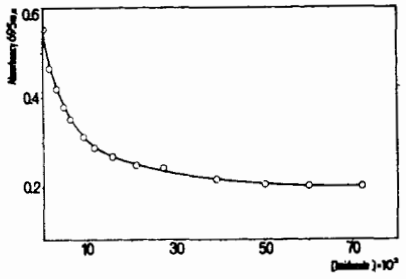

FIGURE 1: The effect of increasing imidazole concentrations on the absorbancy at 695 mμ of 7.0×10^{-1} mM ferric cytochrome *c*, 0.1 M phosphate buffer, pH 7.4; 24°.

Results

Titration of Ferricytochrome c with Imidazole. The effect of increasing imidazole concentrations on the optical density of cytochrome *c* at 695 mμ is shown in Figure 1. Using the initial and final values, corresponding to the free and saturated hemoprotein, the fractions of free and combined cytochrome *c* were estimated, and the logarithms of their ratios were drawn against the logarithms of imidazole concentrations. Figure 2 shows a typical titration at pH 7.4 and 24°. The slope of the line is 1.08, indicating a 1:1 stoichiometry. From titrations of this type, the binding constants could be determined. This was done at several pH values and it was found that the observed constants, K_{obsd}, estimated on the basis of total imidazole added, increased with increasing pH. However, when the constants were estimated on the basis of the concentration of unprotonated imidazole, they remained unchanged, as shown in Figure 3. This implies that only unprotonated imidazole is bound by cytochrome *c*. At pH values below the p*K* of the ligand, the liberated proton appears as a product of the reaction, lowering the observed equilibrium constant. The same pH effect is observed in the reaction between ferricytochrome *c* and cyanide (George *et al.*, 1967). At pH 7.4, titrations were done at 18 and 32°. pH values were measured all along the titrations, and the fractions of unprotonated imidazole were estimated after correcting the p*K* of imidazole for its thermodynamic heat of ionization. This procedure implies the assumption of a heat of ionization constant

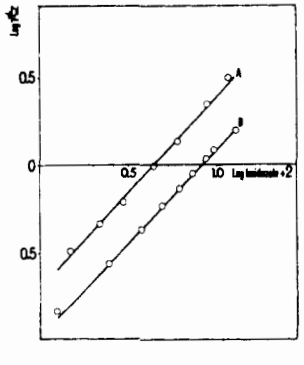

FIGURE 2: A Hill plot of the titrations of ferric cytochrome *c* with imidazole (curve A) or 1-methylimidazole (curve B). Measurements performed at 695 mμ, conditions as in Figure 1.

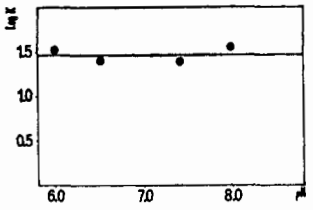

FIGURE 3: The pH dependence of the logarithm of the stability constant of ferricytochrome *c*–imidazole at 24°, phosphate buffers, 0.1 M. Stability constants estimated assuming pK = 6.95 for ionization of imidazole.

with ionic strength that should not introduce undue errors within the range of ionic strength of these titrations.

The values of K obtained at the two temperatures, and the observed thermodynamic parameters derived from them, are listed in Table I. Similar titrations were performed with 1-methylimidazole. At pH 7.4 and 20°, a slope of 1.08 was obtained and the K_{obsd} was estimated as 12 M^{-1} (Figure 2).

The Reduction of Cytochrome c–Imidazole. The reduction of ferric cytochrome *c*–imidazole with solid dithionite was a very fast reaction. Evidence for the formation of a reduced intermediate of the type of ferrocytochrome *c*–cyanide (George and Schejter, 1964) was searched spectrophotometrically. The spectrum of the reduced cytochrome *c* formed in the presence of imidazole was recorded immediately after the addition of the dithionite. The only change that could be observed with respect to native ferrocytochrome *c* was a small increase of about 2% in absorbancy at the peak of the Soret band between 414 and 418 mμ. The absorbancy decreased rapidly to that of native cytochrome *c*. The rate of this change was measured at 416 mμ as described under Methods. Two typical experiments, at pH 7.0, are shown in Figure 4. The reaction followed first order, with rate constants of 2.6×10^{-2} and 1.08×10^{-1} sec^{-1} at 14 and 21°, respectively. From these data, an activation energy of 21.8 kcal/mole was calculated. It was also found that, contrary to the case of cytochrome *c*–cyanide, ascorbate was effective as a reducing agent of ferricytochrome *c*–imidazole. At imidazole concentrations up to 1.4 M, the reduction proceeded with first-order behavior. This concentration range can be divided into two regions. In the first, up to about 0.1 M imidazole, the observed first-order rate constants for a given concentration of ascorbate were identical with those obtained for the free enzyme. It should be

TABLE I: Thermodynamic Parameters for the Stability Constant of Ferricytochrome *c*–Imidazole (pH 7.4).

Temp (°C)	K (M^{-1})	ΔF (kcal/mole)	ΔH (kcal/mole)	ΔS (eu)
18	29.0	−1.94		
			+1.1	+10.2
32	31.6	−2.08		

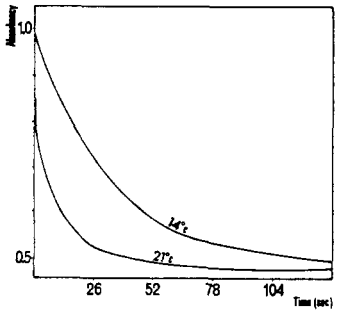

FIGURE 4: The variation with time of the absorbancy at 416 mμ of ferrocytochrome *c*–imidazole (for details, see text).

noticed that this region covers up to 60% of the formation of the cytochrome *c*–imidazole complex, as measured by the disappearance of the 695-mμ band. Above 0.1 M imidazole, the observed first-order rate constants decreased upon increasing the concentration of imidazole, as shown in Figure 5. Thus, this effect of imidazole on the kinetics of the reduction appears to proceed independently from the iron binding reaction. It is possible to ascribe this secondary effect of imidazole to a nonspecific denaturation of the protein. In order to test for this assumption, another denaturing agent, guanidine hydrochloride, was substituted for imidazole. In this case, however, the observed rate constants for reduction with ascorbate increased with increasing guanidine hydrochloride concentrations (Figure 5).

Spectrum of Ferricytochrome c–Imidazole. The effect of imidazole on the cytochrome *c* spectrum in the visible and Soret regions is rather small; the changes can be magnified, though, by determining the difference spectrum of imidazole-saturated cytochrome *c vs.* the free enzyme, using large concentrations of the hemoprotein. A difference spectrum is shown in Figure 6, together with difference spectra of cytochrome *c* in 4.4 M urea, cytochrome *c* at 50°, and cytochrome *c* polymers (Margoliash and Lustgarten, 1962), all measured *vs.* the native, monomeric enzyme at 20°.

Discussion

The shape of the titration curve of ferricytochrome *c* with imidazole followed at 695 mμ suggests that only 1 equiv of ligand is bound per equiv of protein. The unit slope of the Hill plot (Figure 2) is not an absolute indication of 1:1 stoichiometry; it could also be due to a reaction involving the binding of several ligands to a number of protein sites thermodynamically independent and with the same affinity for imidazole. This would agree with the hypothesis that the effect of imidazole is that of a nonspecific denaturing agent. A number of observations speak against this view. First, in general, nonspecific denaturations are the result of cooperative effects of denaturing agents, evidenced by Hill plots with $n > 1$. In the particular case of cytochrome *c*, this is typified by the titration of the effect of guanidine hydrochloride on the 695-mμ band (Schejter and George, 1964). Furthermore, although guanidine hydrochloride

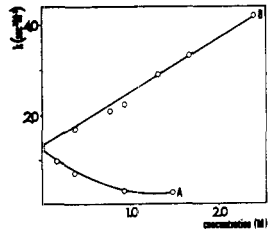

FIGURE 5: The effect of imidazole (curve A) and guanidine (curve B) on the rate of reduction of ferricytochrome *c* with ascorbate; 0.04 M phosphate buffer, pH 7.1; I = 2.88 (NaCl + ligand); 24°.

is known to be a very strong denaturing agent, its effect on cytochrome *c* does not show up until a concentration of the order of 2 M is reached, and the same is true for urea, while imidazole is already effective at concentrations 100 times smaller. It should be noticed that at imidazole concentrations above 0.1 M, there is a distinct effect on the kinetics of reduction of the enzyme by ascorbate. This effect is in the opposite direction to the effect of guanidine (Figure 5), showing again that the two molecules act differently on cytochrome *c*. It is possible that the effect of imidazole on the kinetics of cytochrome *c* reduction is due to a binding of the ligand to a side chain of the protein. In fact, a reaction of this type has been described by Shinitzky *et al.* (1966), who found that imidazole binds lysozyme by forming a charge-transfer complex with a tryptophan residue. Cytochrome *c* possesses a single tryptophan at position

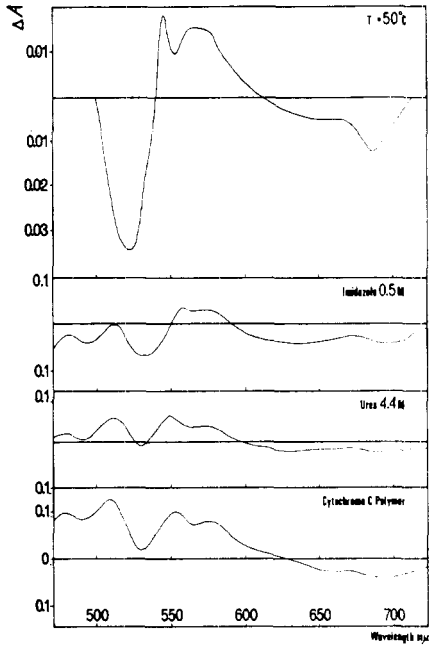

FIGURE 6: Difference spectra of (top to bottom): 1.67×10^{-4} M ferricytochrome *c*, 50°; 1.14×10^{-4} M ferricytochrome *c*–imidazole (ligand concentration 0.5 M); 1.5×10^{-4} M ferricytochrome *c* in 4.4 M urea; 1.12×10^{-4} M ferricytochrome *c* polymers. All samples measured at pH 7.4 against equivalent concentrations of native ferricytochrome *c* at 20°.

59 that appears in all the species investigated and may therefore be involved in electron-transport processes.

The denaturing agents act similarly on cytochrome *c*, regardless of the fact that guanidine hydrochloride is present as guanidinium ion, while urea is present as an uncharged species. Imidazole, on the other hand, binds cytochrome *c* in its unprotonated form only, as shown by the experiment of Figure 3. In fact, the pH dependence of the equilibrium constant in this pH range is in keeping to what should be expected from our knowledge of the pH dependence of the equilibrium between cytochrome *c* and ligands such as cyanide and azide (George *et al.*, 1967).

Thus, we feel justified in treating the reaction of ferricytochrome *c* with imidazole as a ligand binding reaction, in which the ligand binds the cytochrome *c* iron. It should be expected that this reaction follow the same pattern of thermodynamic parameters observed in the other reactions between ferricytochrome *c* and iron ligands (George and Tsou, 1952; Margoliash and Schejter, 1966). This is actually so; at neutral pH the heat of the reaction is 1.1 kcal/mole and the favorable free-energy change is due to an over-all increase in entropy. Furthermore, the contribution to the entropy change due to changes in the protein, $S_{ML} - S_M$, may be evaluated from the sum $\Delta S + S_L$, where S_L is the entropy of formation of aqueous imidazole; S_M and S_{ML} are the entropies of formation of the free and bound protein, respectively; and ΔS is the observed entropy of the reaction (George, 1956). Using empirical equations (Cobble, 1953), S_L for imidazole may be estimated at 32 eu, thus giving a value of $S_{ML} - S_M = 42.2$ eu. This value is only two-thirds of the value obtained for the reaction with cyanide, 60 eu (George and Tsou, 1952). However, its importance should be judged only in qualitative terms, since the different ionic strength conditions of the measurements preclude an exact thermodynamic comparison.

The major obstacle to our interpretation is the fact that the rate of the reaction is practically independent of the concentration of imidazole (Greenwood and Palmer, 1965), while the rate of formation of ferricytochrome *c*–cyanide increases linearly with the concentration of the ligand (George and Tsou, 1952). Nevertheless, it seems to us that the difference between the kinetics of the two reactions is one of degree rather than kind. Greenwood and Palmer (1965) stated that the observed rate was invariant at 0.49 sec^{-1}, for imidazole concentrations of 5–50 mM, but that it increased 4-fold over a 100-fold range of imidazole concentration. Now, for the reaction approaching equilibrium, $k_{obsd} = k_f[Im] + k_b$, where k_{obsd} is the observed rate constant, while k_f and k_b are the true rate constants for the formation and the dissociation of the complex, respectively. In this particular reaction, the equilibrium constant is about 30; thus, k_f is only one order of magnitude larger than k_b. Under these conditions, a tenfold increase in imidazole concentration should not affect greatly the observed rate constant, causing the reaction rate to be apparently independent of the concentration of the ligand, especially when these concentrations are small.

The fact that imidazole can act as a ligand for the ferricytochrome *c* iron should not be surprising, since ferric heme proteins such as hemoglobin are known to form imidazole complexes (Scheler *et al.*, 1957). It should be expected that the protein group displaced from iron coordination be the same whether the ligand is cyanide or imidazole. In the case of cyanide, the available evidence indicates that the displaced group is the sulfur atom of methionine-80 (Harbury *et al.*, 1965). The crystallographic analysis of horse ferricytochrome *c* at 4-Å resolution points to methionine-80 as a very plausible iron ligand (Dickerson *et al.*, 1967). The binding of *N*-acetylmethionine to heme peptides causes the appearance of a band near 695 mμ (Shechter and Saludjian, 1967). Furthermore, the 695-mμ band of ferricytochrome *c* is due to a transition polarized perpendicularly to the porphyrin ring (Eaton and Hochstrasser, 1967). The spectroscopic facts agree, therefore, with a structure of ferricytochrome *c* involving binding of the iron to the sulfur of methionine-80, although, as pointed out by Eaton (1967), it does not necessarily imply that the 695-mμ band arises from a sulfur to iron, or iron to sulfur, charge-transfer transition.

In view of these facts, the disappearance of the 695-mμ band is supporting evidence for the hypothesis that imidazole displaces the methionine-80 sulfur from iron coordination. It is of interest, in this respect, to compare the various difference spectra shown in Figure 6. Their essential features are strikingly similar. In addition to the expected trough in the 650–700-mμ region, there is a less marked trough between 600 and 650 mμ; two peaks, at 575 and 550 mμ; a trough at 530 mμ; and another at 490 mμ. By purely empirical analogy, it may be tentatively suggested that the iron binding sulfur of native ferricytochrome *c* is replaced by a nitrogenous ligand when the molecule is heated, denatured by urea, or converted into a dimer. This conclusion, however, is complicated by the spectroscopic similarity between ferrocytochrome *c* and the compound resulting from reduction of ferricytochrome *c* imidazole with dithionite. The only effect observed, at the peak of the Soret band, is indeed small, but it was found to be entirely reproducible. Furthermore, the spectrum is converted into that of ferrocytochrome *c* with kinetic parameters comparable with those of the dissociation of ferrocytochrome *c* cyanide (George and Schejter, 1964). This may indicate that the changes observed at the Soret band peak are due to the dissociation of ferrocytochrome *c*–imidazole. However, since no other differences were detected in the rest of the spectrum, the implication of this interpretation is that native ferrocytochrome *c* and its imidazole complex have almost identical spectra. In this regard it is of interest to point out that the complexes formed by addition of imidazole to carboxymethylated ferrocytochrome *c* (Schejter, 1966) or to ferrocytochrome *c*–heme peptides (Harbury and Loach, 1960), as well as the complex formed by addition of *N*-acetylmethionine to ferrocytochrome *c*–heme peptides (Harbury *et al.*, 1965), have spectra identical with those of the native enzyme, at least in the position of their maxima. Hence, it appears possible that, if the methionine-80 sulfur is an iron ligand

also in reduced cytochrome *c*, its replacement by an imidazole group should not be spectroscopically detectable.

It is of interest that binding by imidazole does not affect the rate of reduction by ascorbate. Since it has been implied that the methionine-80 region is involved in the oxidation of cytochrome *c* by cytochrome oxidase (Margoliash, 1966), our results could well indicate that electron transfer to the iron of ferricytochrome *c* follows a different pathway than electron transfer from the iron of ferrocytochrome *c*. The model of ferric cytochrome *c* (Dickerson *et al.*, 1967) shows that one edge of the porphyrin ring is accessible without interference of the protein chain. Thus, an electron donor may reach by sidewise attack the π-electron system of the porphyrin, and the electron conducted from this porphyrin edge to the iron orbitals, and from them to cytochrome oxidase *via* the methionine-80 sulfur. Attempts to measure the reduction of ferricytochrome *c*–imidazole by an enzymic system, succinic oxidase, were unsuccessful, but this negative result cannot be taken as evidence against our hypothesis due to the large concentration of imidazole required to saturate cytochrome *c*. In any event, if the proposed electron pathways were the same in the oxidation and reduction of cytochrome *c* within the respiratory chain, then the requirement of molecular rotations of cytochrome *c* in order to serve as an electron carrier (Chance and Williams, 1956) would be no longer necessary, and a rigid electron-transport chain could be entirely satisfactory.

References

Chance, B., and Williams, G. R. (1956), *Advan. Enzymol. 17*, 65.

Cobble, J. M. (1953), *J. Chem. Phys. 21*, 1451.

Dickerson, R. E., Kopka, M. L., Weinzierl, J., Varnum, J., Eisenberg, D., and Margoliash, E. (1967), *J. Biol. Chem. 242*, 3015.

Eaton, W. A. (1967), Ph.D. Thesis, University of Pennsylvania, Philadelphia, Pa.

Eaton, W. A., and Hochstrasser, R. M. (1967), *J. Chem. Phys. 46*, 2533.

George, P. (1956), *in* Currents in Biochemical Research, Green, D. E., Ed., New York, N. Y., Interscience, p 338.

George, P., Glauser, S. C., and Schejter, A. (1967), *J. Biol. Chem. 242*, 1960.

George, P., and Schejter, A. (1964), *J. Biol. Chem. 239*, 1504.

George, P., and Tsou, C. L. (1952), *Biochem. J. 50*, 440.

Greenwood, C., and Palmer, G. (1965), *J. Biol. Chem. 240*, 3660.

Harbury, H. A., Cronin, J. R., Fanger, M. W., Hettinger P. T., Murphy, A. J., Myer, Y. P., and Vinogradov, S. N. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1658.

Harbury, H. A., and Loach, P. A. (1960), *J. Biol. Chem. 235*, 3640.

Margoliash, E. (1966), *in* Hemes and Hemoproteins, Chance, B., Estabrook, R. W., and Yonetani, T., Ed., New York, N. Y., Academic, p 371.

Margoliash, E., and Lustgarten, J. (1962), *J. Biol. Chem. 237*, 3397.

Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113.

Margoliash, E., and Walasek, O. F. (1967), *Methods Enzymol. 10*, 339.

Schejter, A. (1966), *in* Hemes and Hemoproteins, Chance, B., Estabrook, R. W., and Yonetani, T., Ed., New York, N. Y., Academic, p 320.

Schejter, A., and George, P. (1964), *Biochemistry 3*, 1045.

Scheler, W., Schoffa, G., and Jung, F. (1957), *Biochem. Z. 329*, 232.

Shechter, E., and Saludjian, P. (1967), *Biopolymers 5*, 788.

Shinitzky, M., Katchalski, E., Grisaro, V., and Sharon, N. (1966), *Arch. Biochem. Biophys. 116*, 332.