Carbon Monoxide as a Probe for Conformation Changes of Ferrocytochrome *c*†

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ABSTRACT: Horse heart ferrocytocytochrome *c* is induced to complex with carbon monoxide at pH 5–7 under the influence of methyl, ethyl, *n*-propyl, isopropyl, and *tert*-butyl alcohols, ethylene and propylene glycols, and urea. The extent of reaction, which involves the displacement of methionine-80 by carbon monoxide is influenced by the quantity and nature of the alcohol. Thus the formation of ferrocytochrome *c*–carbon monoxide is favored by increasing alcohol chain length but branched-chain alcohols and the glycols are relatively less effective. The reaction of carbon monoxide with ferrocytochrome *c* is initiated at lower alcohol concentrations than those inducing autoxidation. The extent of ferrocytochrome *c*–carbon monoxide formation, induced by urea, varies in a two-step manner with the stages centered at 3.6 and 6.5 M urea. A slow rate of attainment of equilibrium for the formation of ferrocytochrome *c*–carbon monoxide was observed in urea and alcoholic solutions. This is probably a consequence of the similar affinities of methionine-80 and carbon monoxide for the heme iron. The formation of the ferrocytochrome *c*–carbon monoxide is reversed by removal of the denaturant and by displacement of the excess carbon monoxide from the solution. Carbon monoxide can thus be used as a probe for relatively small conformational changes in the heme crevice of ferrocytochrome *c*.

Ferrocytochrome *c*, in its native state, does not react with carbon monoxide, in contrast to many other hemoproteins and Fe^{2+} porphyrins (Butt and Keilin, 1962). This failure to react is a consequence of the strength of the histidyl and methionyl iron(II) ligands and the sterically hindered situation of the iron(II) in the closed crevice structure of the protein. At extremes of pH (above pH 12 and below pH 4), however, ferrocytochrome *c* does react with carbon monoxide, but the resultant carbon monoxide–ferrocytochrome *c* complex is dissociated on neutralization. The study of the kinetics of this dissociation has yielded valuable information on the crevice structure of cytochrome *c* (George and Schejter, 1964).

Irreversible denaturation of cytochrome *c* by incubation in 0.6 N KOH for 24 hr followed by neutralization yielded a cytochrome *c* molecule which reacted readily with carbon monoxide. It was consequently proposed that the reaction of ferrocytochrome *c* with carbon monoxide could be used as a probe for detection of denaturation of the protein (Ben-Gershom, 1961).

In our earlier attempts to elucidate structure–function relationships of cytochrome *c* by modification of the native conformation with alcohols and urea and studying the properties of the modified protein, we demonstrated that only minor conformational changes, which could not be detected by circular dichroism spectropolarimetry, considerably alter the reactivity of ferrocytochrome *c* with oxygen (Kaminsky *et al.*, 1972; Kaminsky *et al.*, 1971; Kaminsky and Davison, 1969; Yong and Kaminsky, 1971). In an effort to extend these studies we have investigated the conformational changes involved when cytochrome *c* becomes autoxidizable in alcoholic and urea solutions by using the carbon monoxide reacting properties of the denatured protein.

In this paper we report on the reaction of ferrocytochrome *c* with carbon monoxide in solutions of varying concentrations of methyl, ethyl, *n*-propyl, isopropyl, and *tert*-butyl alcohols, ethylene and propylene glycols, and urea. Control studies have also been performed on the ferroheme undecapeptide from cytochrome *c*.

Experimental Section

Materials. Horse heart cytochrome *c* (Grade 1, batch 724, purity 93.8%, iron content 0.412%) was obtained from Miles-Seravac Ltd. and reduced by the method of Yonetani and Ray (1965).

Alcohols were British Drug Houses' Analar Grade and were used without further purification. Urea was Merck crystalline extra pure grade. Water was glass-distilled and deionized.

Carbon monoxide (99.5% minimum purity) was supplied by Matheson Gas Products and was washed with a 10% triacetoxybenzene solution in 15% potassium hydroxide before use.

The heme undecapeptide was prepared by peptic digestion of cytochrome *c* by the method of Harbury and Loach (1960) with a final purification using a column (2.5 × 30 cm) of Sephadex G-25, using water as eluent.

Methods. Cytochrome *c* concentrations throughout this study varied between 2.41 and 2.84 × 10^{-4} M and were determined using molar extinction coefficient differences at 550 nm of $\Delta\epsilon_{550}$ (reduced − oxidized) = 21.3 mM^{-1} cm^{-1} (Margalit and Schejter, 1970).

Solutions of alcohols and urea were made up as previously described (Kaminsky *et al.*, 1971). Spectra were determined using a Unicam SP-8000 spectrophotometer fitted with a Beckman 10-in. recorder or a Unicam SP-1800 spectrophotometer. The cells used were modified Thunberg cuvets, the tops of which were adapted to accommodate rubber seals (serum caps).

Oxygen-free nitrogen was bubbled for a minimum of 30 min through 2.54 ml of alcoholic or urea solution in the Thun-

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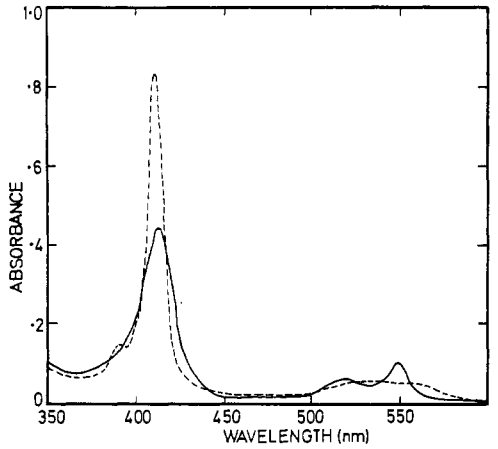

FIGURE 1: Absorption spectra of ferrocytochrome *c* (—) and ferrocytochrome *c*–carbon monoxide (---) in 15.6 mole % ethanol; cytochrome *c* concentration, 2.8 μM; acetate buffer, 0.1 M, pH 5.

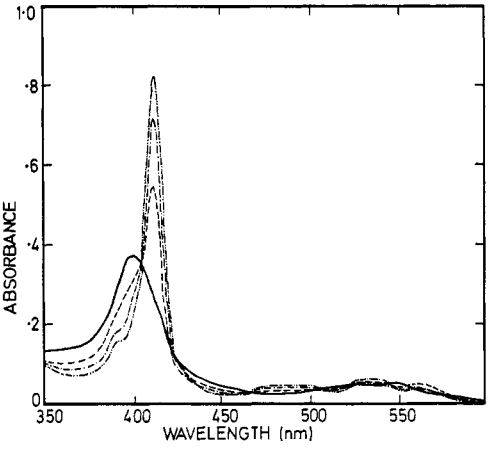

FIGURE 2: Absorption spectra of ferrocytochrome *c* (—) and ferrocytochrome *c*–carbon monoxide immediately after the addition of carbon monoxide (---), 10 min after addition of carbon monoxide (-·-), 40 min after addition of carbon monoxide (-···-), in 24 mole % ethanol, other conditions as in Figure 1.

berg cuvet. A trace of dithionite was added to an aqueous solution of ferrocytochrome *c* to ensure complete reduction and 0.05 ml of this solution was added to the deoxygenated solution in the cuvet through the rubber seal. After the spectrum of the reduced cytochrome *c* was recorded, oxygen-free carbon monoxide was bubbled through the solution for 5 min. The spectrum was then immediately recorded and scans were made at 10-min intervals until no change was observed between any scan and the previous one.

The relative change in Soret absorbance induced by the carbon monoxide, $\Delta\epsilon/\epsilon_0$ was plotted against mole % alcohol or urea molarity. ϵ_0 is the Soret molar extinction coefficient of ferrocytochrome *c* in aqueous solution under the conditions of the experiment, and $\Delta\epsilon_0$ is the difference between the Soret molar extinction coefficients of ferrocytochrome *c* and ferrocytochrome *c*–carbon monoxide in the particular alcoholic or urea solution.

Equilibrium constants for the transition native ferrocytochrome *c* $\rightleftharpoons$ ferrocytochrome *c*–carbon monoxide were calculated from the relationship $K = (A^{obsd} - A^{nat})/(A^{CO} - A^{obsd})$, where A^{nat} is the Soret absorbance of native ferrocytochrome *c*, A^{CO} is the similar absorbance of ferrocytochrome *c*–carbon monoxide, and A^{obsd} is the similar absorbance of the partially formed carbon monoxide complex of the protein Calculation of the equilibrium constants is based on the following assumptions. A solution of ferrocytochrome *c* which shows no change in its spectrum from 350 to 600 nm after the passage of carbon monoxide through the solution is assumed to be in its fully native conformation while the solution demonstrating the greatest change in Soret absorbance in a particular series of experiments is assumed to be fully complexed with carbon monoxide.

Results

Carbon monoxide produces no change in the visible absorbance spectrum of aqueous solutions of ferrocytochrome *c* at pH 5 except for a Soret peak absorbance increase of at most 0.02 which probably arises from the presence of a small quantity of denatured protein in the sample. This absorbance change is insignificant when compared to carbon monoxide induced absorbance changes of 0.5 which were subsequently obtained in alcoholic solutions. When carbon monoxide was bubbled through alcoholic solutions of ferrocytochrome *c*, however, a number of changes in the spectrum were observed, which became more pronounced as the alcohol content of the solution increased. The absorbance of the Soret peak increased dramatically and its wavelength shifted to 412 nm, while the absorbance bands of ferrocytochrome *c* at 520 and 550 nm were diminished to yield ultimately a spectrum with rather diffuse maxima at about 530 and 562 nm. At higher alcohol concentrations a weak shoulder appeared at about 392 nm (Figure 1). In all cases, except for the glycols, the carbon monoxide induced Soret absorbance increases reached a maximum and then decreased as the alcohol concentrations were further increased. No similar effect was noted with ethylene glycol or propylene glycol, possibly because the concentrations used were not high enough.

The spectral changes observed in solutions of low alcohol concentration rapidly reached equilibrium after the passage of carbon monoxide for 5 min while at the higher alcohol concentrations these changes occurred slowly and stable spectra were only observed approximately 40 min after the bubbling of the gas (Figure 2). This rate of spectral change was particularly slow at the highest alcohol concentrations studied (60% v/v).

In contrast to the marked enhancing effect of alcohols on the Soret absorbance of ferricytochrome *c* (Kaminsky and Davison, 1969) the Soret peak of ferrocytochrome *c* was virtually unchanged by the alcohols except for a marked change at very high concentrations. Thus, for example, in 27 mole % ethyl alcohol, 23 mole % *n*-propyl alcohol, and 19 mole % *tert*-butyl alcohol the spectrum of ferrocytochrome *c* was altered to more closely resemble that of the oxidized form of the protein with the Soret absorbance peak shifted to 402 nm and having the 520- and 550-nm absorbance maxima replaced with a diffuse band of greatly diminished absorbance having a weak maximum at 550 nm. When carbon monoxide was bubbled through these solutions, however, the spectra were slowly altered to resemble ultimately those of ferrocytochrome *c*–carbon monoxide obtained at lower alcohol concentrations (Figure 2). All spectra of the ferrocytochrome *c*–carbon monoxide compound immediately reverted to those of ferricytochrome *c* after the passage of oxygen through the solution.

The plot of $\Delta\epsilon/\epsilon_0$ against concentration of alcohol (Figure

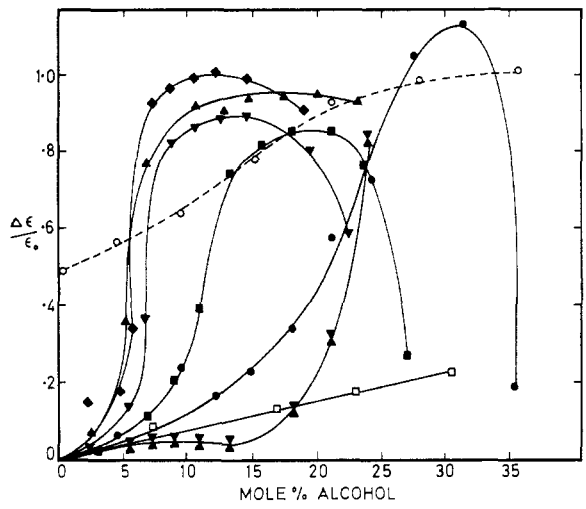

FIGURE 3: Effect of alcohols on the Soret absorbance of ferrocytochrome *c* after treatment with carbon monoxide. The relative change in molar absorptivity, $\Delta\epsilon/\epsilon_0$ is plotted as a function of alcohol concentration. (●) Methyl, (■) ethyl, (▲) *n*-propyl, (▼) isopropyl, (◆) *tert*-butyl alcohols, (□) ethylene, and (⧗) propylene glycols; acetate buffer 0.1 M, pH 5. (O---O) effect of methanol on the ferroheme undecapeptide–carbon monoxide complex, phosphate buffer 0.1 M, pH 7.

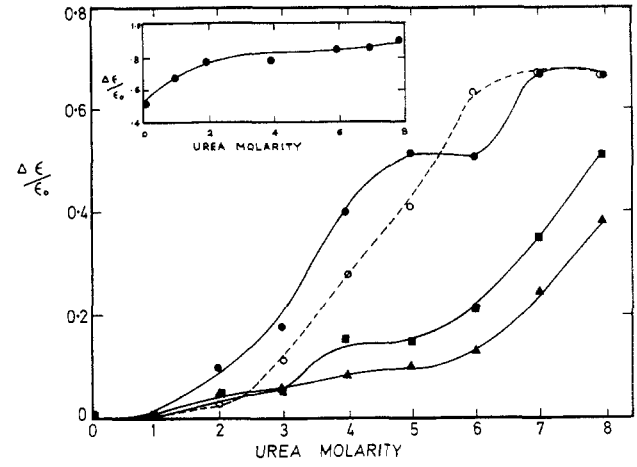

FIGURE 5: Effect of urea on the Soret absorbance of ferrocytochrome *c* after treatment with carbon monoxide. The relative change in molar absorptivity is plotted against urea concentration. (●) pH 5, (■) pH 6, (▲) pH 7, and (O) pH 5 with glycerol (10% v/v) added; acetate buffer 0.1 M at pH 5 and 6, phosphate buffer 0.1 M at pH 7. Inset: effect of urea on the ferroheme undecapeptide–carbon monoxide complex, other details as above.

3) shows the increasing effectiveness of the alcohols as denaturants with an increase in hydrocarbon chain length. Thus 21 mole % of methyl alcohol was required to produce 50% of the maximum change in the Soret absorbance while only 12.6 mole % ethyl alcohol and 6.5 mole % *n*-propyl alcohol were required to produce the same effect. Branching of the chain decreased the effectiveness of the alcohol as a denaturant and thus 8.1 mole % isopropyl alcohol was required to produce 50% of the maximum change as compared to 6.5 mole % of *n*-propyl alcohol. Ethylene glycol and propylene glycol were also dramatically less effective as denaturants than ethyl and *n*-propyl alcohols.

Plots of the logarithms of the equilibrium constants for ferrocytochrome *c* (native) $\rightleftharpoons$ ferrocytochrome *c*–carbon monoxide against alcohol concentrations up to those producing the maximum absorbance change (Figure 4) indicate that the reaction possibly occurs in three stages. There is initially only a slight enhancement of the carbon monoxide induced absorbance change with increasing alcohol concentration followed by a rapidly increasing effect and finally a slowly changing increase up to the maximum effect. The subsequent decrease in the Soret absorbance of ferrocytochrome *c*–carbon monoxide at still higher concentrations of alcohols suggests a fourth stage in the reaction which possibly involves an increase in the helix content of the protein.

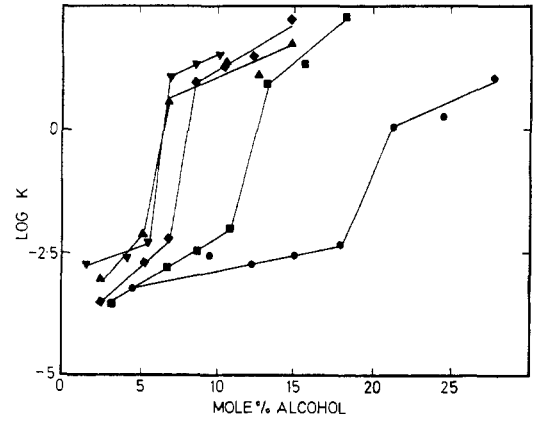

FIGURE 4: Effect of alcohols on the Soret absorbance of ferrocytochrome *c* after treatment with carbon monoxide. The logarithm of the equilibrium constant for ferrocytochrome *c* (native) $\rightleftharpoons$ ferrocytochrome *c*–carbon monoxide against alcohol concentration. (●) Methyl, (■) ethyl, (▲) *n*-propyl, (◆) isopropyl, and (▼) *tert*-butyl alcohols; other conditions as in Figure 4.

Similar effects were noted at pH 6 but a larger concentration of alcohol was required to enable the carbon monoxide to undergo an equivalent reaction. Thus at pH 5, 6.5 mole % of *n*-propyl alcohol was required to produce 50% of the maximum spectral change while at pH 6 10 mole % was required. The maximum spectral change at pH 6 was however equal to that at pH 5.

Carbon monoxide also reacted with ferrocytochrome *c* in aqueous solutions of urea. The resulting spectral changes were identical with those produced by carbon monoxide in aqueous alcoholic solutions. The slow rates of the spectral changes after the passage of carbon monoxide were again observed and were particularly marked at higher urea concentrations.

The results obtained with urea as a denaturant are shown in Figure 5. The ferrocytochrome *c* Soret absorbance increases with increasing urea concentration at pH 5 occurred in two stages with centers at approximately 3.6 and 6.5 M urea and with the maximum effect being observed at 7 M urea. A similar two-stage change was obtained at pH 6 with the first stage also occurring with its center at 3.6 M urea. The maximum was, however, apparently not reached in 8 M urea, which was the highest concentration obtainable under the condition of the experiment, and thus the second stage of urea denaturation appears to have been inhibited at pH 6. The carbon monoxide induced enhancement of the Soret absorbance throughout the range of urea concentrations was markedly less at pH 6 than at pH 5. This reduction in the effect of carbon monoxide on the spectrum of ferrocytochrome *c* in urea solution was even more marked at pH 7 where in 8 M urea the ferrocyto-

chrome *c*–carbon monoxide Soret absorbance appeared to be only approximately half-developed. At pH 7 the 3.6 M urea change which was observed at pH 5 and 6 had virtually disappeared. The effects were considerably altered when glycerol (10% v/v) was added to the urea solutions at pH 5. At urea concentrations of up to 5 M the Soret absorbance increases induced by carbon monoxide were diminished by the glycerol. Between 5 and 7 M urea the effects were enhanced by the glycerol, and at 7 and 8 M urea, glycerol produced no differences in the spectra. Glycerol also increased the rate of interaction of carbon monoxide with ferrocytochrome *c*; thus in 7 M urea at pH 5 the spectrum reached its final state 40 min after the addition of carbon monoxide while in the presence of glycerol a stable spectrum was obtained after only 10 min under otherwise identical conditions.

The reaction of carbon monoxide with ferrocytochrome *c* under denaturing conditions was shown to be reversible. When ferrocytochrome *c*–carbon monoxide in 6 M urea solution at pH 5 was diluted with anaerobic, carbon monoxide saturated acetate buffer at pH 5 so as to reduce the final urea concentration to 3 M, the Soret absorbance was decreased to that previously observed in 3 M urea, which indicates a reversal of the effect in 6 M urea. This experiment thus also indicates the reversible nature of the urea denaturation of ferrocytochrome *c*. The carbon monoxide induced absorbance increases of ferrocytochrome *c* could also be reversed by the removal of the carbon monoxide with a stream of nitrogen. Thus when nitrogen was passed anaerobically through a 6 M urea solution of ferrocytochrome *c*–carbon monoxide for 15 min the spectrum completely reverted to that of ferrocytochrome *c* obtained prior to the passage of carbon monoxide. When, however, nitrogen was bubbled for only 5 min and the cuvet was then resealed, the spectrum had undergone only partial reversion which continued to change slowly back to that of the ferrocytochrome *c*.

Aqueous solutions of the ferroheme undecapeptide of cytochrome *c* reacted with carbon monoxide under anaerobic conditions. The resultant enhanced Soret absorbance was further increased by the presence of alcohols (Figure 3) and urea (Figure 5). The reaction with carbon monoxide was relatively fast and apparently completed before the end of the 5-min bubbling period with carbon monoxide.

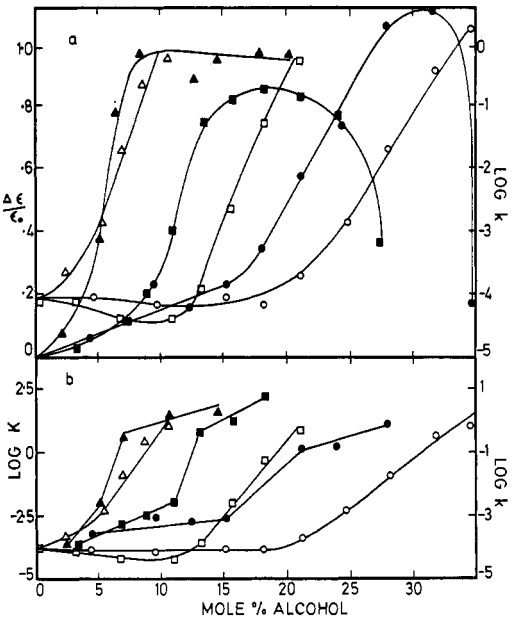

FIGURE 6: Comparison of the effects of alcohols on autoxidation rates and carbon monoxide reactions of ferrocytochrome *c*. Autoxidation rates in (○) methyl, (□) ethyl, and (△) *n*-propyl alcohols. (a) Carbon monoxide included Soret absorbance changes in (●) methyl, (■) ethyl, and (▲) *n*-propyl alcohols. (b) Equlibrium constants for cytochrome *c* ⇋ cytochrome *c*–carbon monoxide in (●) methyl, (■) ethyl, and (▲) *n*-propyl alcohols. Carbon monoxide reaction data were taken from Figures 3 and 4; autoxidation data were taken from Kaminsky *et al.* (1971).

Discussion

The spectra of the ferrocytochrome *c*–carbon monoxide complex in aqueous solutions of alcohols and urea at pH 5 are virtually identical with those obtained in aqueous solution at pH 14 (Butt and Keilin, 1962), at pH 2.4 (Kurihara and Sano, 1970), and for irreversibly denatured cytochrome *c* at pH 7 (Ben-Gershom, 1961). This implies that the central coordination complexes formed under these differing conditions are similar and that the roles of urea and alcohols are simply those of denaturing agents.

The most likely function of alcohols in inducing ferrocytochrome *c*–carbon monoxide formation is a stabilization of the open heme crevice structure of the protein with subsequent weakening of the native heme iron ligand bonds and exposure of the heme iron to carbon monoxide. This is borne out by the enhanced effectiveness of the more hydrophobic alcohols in inducing complex formation (Figure 3), in view of the known hydrophobicity of the heme crevice (Dickerson *et al.*, 1971). The diminished effectiveness of ethylene glycol and propylene glycol compared to ethyl alcohol and *n*-propyl alcohol in inducing complex formation with carbon monoxide is further evidence for the role of hydrophobicity in the crevice disrupting properties of the alcohols.

The alcohol-induced conformational changes of ferrocytochrome *c*, which are sufficient to enable carbon monoxide to complex with the heme iron, are apparently relatively minor. This follows from the fact that no changes in the circular dichroism spectrum were detected in alcoholic solutions of ferrocytochrome *c* identical with those permitting complex formation with carbon monoxide (Kaminsky *et al.*, 1972). These solvent-induced conformational changes are, however, sufficient to permit autoxidation of ferrocytochrome *c* (Kaminsky *et al.*, 1971).

The relative effects of alcohols on the rates of autoxidation and the carbon monoxide induced Soret absorbance increases of ferrocytochrome *c* are shown in Figure 6a. It is apparent that the onset of the reaction with carbon monoxide occurs at lower alcohol concentrations than the onset of autoxidation. This is more clearly demonstrated in Figure 6b where a comparison of the equilibrium constants for the carbon monoxide reaction and the rate constants for the autoxidation reveal that the initiation of autoxidation appears to correspond only to the second phase of the carbon monoxide reaction. This is contrary to the suggestion of Butt and Keilin (1962), that the formation of ferrocytochrome *c*–carbon monoxide is not as reliable an indication of denaturation as is autoxidation. When ferrocytochrome *c* was denatured by benzoate (Kazero and Tsushima, 1961) or by conditions of low pH (Chance, 1961), autoxidation was again seen to precede reaction with carbon monoxide in contrast to our results. It is thus apparent that the mode of denaturation plays some role in determining the relative ease of reaction with carbon monoxide or oxygen and that availability of the reagents to the heme iron cannot be the major factor. It might be expected that interaction with

carbon monoxide would follow autoxidation since the former requires displacement of the methionine ligand while the latter does not. However stereochemical differences resulting from the C–O bond being perpendicular to the heme plane in hemeproteins while the O–O is either bent from perpendicular of parallel, when bound to the heme (Caughey *et al.*, 1965), might play a role in reversing the order of reaction.

The slow rate of attainment of equilibrium for the formation of ferrocytochrome *c*–carbon monoxide, as evidenced by Soret band absorbance changes, is not a consequence of slow urea- and alcohol-induced conformational changes since preincubation of the protein in the denaturants did not alter this rate. In the absence of denaturants the carbon monoxide is apparently not available to the heme iron and thus the equilibrium is shifted almost completely to the left. As increasing quantities of urea or alcohol are added the open crevice form is stabilized, which makes the carbon monoxide available at the site of reaction and weakens the iron–methionine bond, both of which would drive the reaction to the right. The removal of urea (by dilution) however, forces the equilibrium back to the left, which possibly indicates that the methionine 80 is so favorably orientated for liganding to the iron that it displaces the carbon monoxide which is present in saturating concentrations. It is thus possible that the relative binding strengths of the carbon monoxide and methionine are similar under the conditions pertaining to these experiments and that the slow attainment of equilibrium is the result of competition between the two relatively fast reactions opposing one another. This is borne out by the fact that removal of all the carbon monoxide by a stream of nitrogen rapidly drives the equilibrium to the left but if small quantities of carbon monoxide are left in the solution then attainment of an equilibrium state is again slow.

The relatively fast reaction of carbon monoxide with the heme undecapeptide, which contains no methionine ligand, corroborates the suggestion that in cytochrome *c* the reaction with carbon monoxide is also rapid but is observed to be slow because of competition with the reverse reaction, since a similar reversal cannot occur with the hemepeptide. The enhancement of the Soret absorbance of heme peptide–carbon monoxide by both methanol and urea possibly results from an alteration in the state of aggregation of the peptide which would alter the position of equilibrium and thus the Soret absorbance.

The markedly reduced effect of the alcohols and urea in modifying the Soret absorbance of native ferrocytochrome *c* as compared with the effects on ferricytochrome *c* (Kaminsky and Davison, 1969) is yet further evidence for the enhanced conformational stability of the reduced molecule.

The two-step effect of urea in inducing ferrocytochrome *c*–carbon monoxide interactions coincides with the two-stage urea-induced denaturation of ferrocytochrome *c* as monitored by circular dichroism spectral changes (Meyer, 1968). Meyer suggests that the first stage corresponds to the displacement of the polypeptide chain from the heme group, *i.e.*, an opening of the heme crevice, while the second stage involves displacement of the methionine-80 ligand and extensive unfolding of the protein chain. Our data appear to support this thesis since it would be expected that opening of the heme crevice, which would make carbon monoxide available to the heme iron, would shift the equilibrium in the direction of the carbon monoxide complexed form of cytochrome *c*. Subsequent urea-induced displacement of the methionine-80 would then shift this equilibrium further in the same direction.

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