Contribution from the Chemistry Departments,
Brookhaven National Laboratory, Upton, New York 11973, and Hofstra University, Hempstead, New York 11550

Kinetics and Mechanism of the Oxidation of Horse Heart Ferrocytochrome *c* by Tris(1,10-phenanthroline)cobalt(III) at Low pH

BRUCE S. BRUNSCHWIG[1a] and NORMAN SUTIN*[1b]

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The kinetics of the oxidation of ferrocytochrome *c* by tris(1,10-phenanthroline)cobalt(III) has been studied by the stopped-flow technique. The oxidation reaction is first order with respect to each reactant. Measurements in chloride media (μ = 0.13 M, 25 °C) over the pH range 1–7 revealed a rate maximum at pH 2.9 ($k = 6.7 \times 10^4$ M^{-1} s^{-1}). By contrast, the rate constants at pH 1 and 5.8 are 3.2×10^4 and 2.1×10^3 M^{-1} s^{-1}, respectively. Below pH 1.7 biphasic kinetics are observed with the slower reaction having a first-order rate constant of ~ 2 s^{-1}, independent of oxidant concentration. The slow process is ascribed to a conformational rearrangement of the ferricytochrome *c* which is produced in the mixed-spin form but rearranges to the more stable high-spin form.

Introduction

Cytochrome *c*, a small heme protein, is a component of the respiratory chain of all aerobic organisms. In vivo the iron in the cytochrome *c* undergoes cyclic oxidation to iron(III) and reduction to iron(II) as electrons are passed from the Krebs cycle down to oxygen. The heme prosthetic group resides in a pocket; the polypeptide chain is wrapped almost entirely around it with only an edge of the heme exposed to the solution. At physiological pH the iron atom lies approximately in the plane of the porphyrin ring with its fifth and sixth coordination positions occupied by a nitrogen ring atom of histidine-18 and the sulfur atom of the methionine-80.[2,3] At low and high pH this structure breaks down.

Despite extensive studies of the redox reactions of cytochrome *c* the exact nature of the electron-transfer reactions in vivo is not known.[4] Studies of the reduction of the ferricytochrome *c* have suggested that both adjacent and remote attack mechanisms are possible with the latter reactions proceeding through the exposed heme edge.[5-7] Studies of the reduction of the iron(III) protein have included a wide pH range in order to understand changes in mechanism and the effect that changes in structure have on the redox reaction.[8] For example, the chromium(II) reduction of the iron(III) can proceed by both an adjacent and a remote pathway.[6] At low pH in chloride media an adjacent pathway is operative but above pH 5.5 the remote pathway predominates.

For ferrocytochrome *c* the heme crevice is less flexible and the iron less accessible due to the strength of the iron–sulfur bond.[9,10] Rapid oxidation of the iron(II) species is therefore expected to proceed by a remote attack of the oxidant. To date no systematic study of the variation in oxidation rate with pH has been undertaken for the iron(II) system. The oxidation of ferrocytochrome by tris(1,10-phenanthroline)cobalt(III) has recently been studied at neutral and basic pH.[11] The present work extends these measurements to pH 1 in order to determine the effect of pH-induced configuration changes on the redox rate.

Experimental Section

Reagent grade chemicals were used throughout except for the potassium biphthalate which was primary standard grade. Horse heart ferricytochrome *c* (type III) was purchased from Sigma and used without further purification. Solutions containing ferrocytochrome c were prepared by adding a pinch of sodium dithionite to aqueous solutions of ferricytochrome *c*.[11] The ferrocytochrome solutions were then loaded onto a 0.7 × 5 cm Bio-Rad 70 cation-exchange column which was washed with approximately 50 ml of water; the ferrocytochrome *c* was then eluted with 3 ml of 1 M sodium chloride. To prepare ferrocytochrome *c* solutions below pH 3, hydrochloric acid was added to deaerated ferrocytochrome *c* solutions in an all-glass system. For solutions of pH >3 the deaerated ferrocytochrome was injected into the deaerated buffer. The tris(phenanthroline)cobalt(III) perchlorate was prepared according to the method of Pfeiffer and Werdelman.[12] The purity of the solid was determined spectrophotometrically using ϵ_{350} 3700 M^{-1} cm^{-1}.[13]

Table I. Second-Order Rate Constants for the Oxidation of Horse Heart Ferrocytochrome *c* by Tris(1,10-phenanthroline)cobalt(III) at 25 °C and μ = 0.13 M

pH	$10^{-4}k$, M^{-1} s^{-1}	pH	$10^{-4}k$, M^{-1} s^{-1}
1.0[a]	3.2	3.4[b]	3.2
1.2[a]	2.6	3.8[b]	1.0
1.3[a]	2.9	4.4[b]	0.39
1.5[a]	3.3	4.8[b]	0.25
1.7[a]	3.4	5.4[b]	0.22
1.9[b]	3.9	5.7[b]	0.21
2.0[a]	4.1	5.7[c]	0.17
2.4[a]	4.6	5.8[b]	0.21
2.6[a]	5.0	5.9[c]	0.18
2.6[b]	6.6	6.2[c]	0.20
2.7[b]	6.7	6.6[c]	0.23
2.9[b]	7.3	6.8[c]	0.22
2.9[b]	6.1	6.9[c]	0.22
3.1[b]	4.9	7.0[c]	0.23
3.4[b]	3.0		

[a] No buffer. [b] Biphthalate buffer. [c] Phosphate buffer.

The oxidation of horse heart ferrocytochrome *c* was studied from pH 1 to 7. For kinetic studies above pH 2.6 the buffer was present in only the cytochrome solution. For studies below pH 2.6 both solutions were brought to the same pH by the addition of hydrochloric acid. For pH's between 2.6 and 5.5 a potassium biphthalate buffer was used with a total phthalate concentration of 0.013 M in the reaction mixture. Above pH 5.5 experiments were performed using a biphthalate or a phosphate buffer in concentrations of either 0.005 or 0.013 M. The ionic strength of each of the solutions was adjusted to 0.13 M by the addition of sodium chloride.

The kinetic measurements were performed on a Durrum stopped-flow spectrophotometer which had been modified as follows. The output from the photomultiplier tube was dropped across a 1-MΩ resistor, offset by a constant voltage, and amplified. The amplified signal was digitized (resolution 1 part in 999) and stored in a 39-point memory with the last 10 points being channels 149–159. The digitized kinetic data were punched onto computer cards and the data were then fitted by a nonlinear least-squares program to either a single or a double exponential decay.[14] The rate constants presented are the averages of 4–10 runs with their standard deviations. All kinetic measurements were carried out under pseudo-first-order conditions with the cobalt(III) complex in excess.

Results

The kinetics of the oxidation of ferrocytochrome *c* by tris(1,10-phenanthroline)cobalt(III) were studied in the pH region 1–7. Above pH 2.3 and below pH 7.0 the first-order plots of the absorbance–time data were linear for more than 90% reaction at all wavelengths used (590, 550, 535, and 520 nm). The second-order rate constants were independent of wavelength and of the concentration of the oxidizing agent but depended upon the pH of the solution. The kinetic data

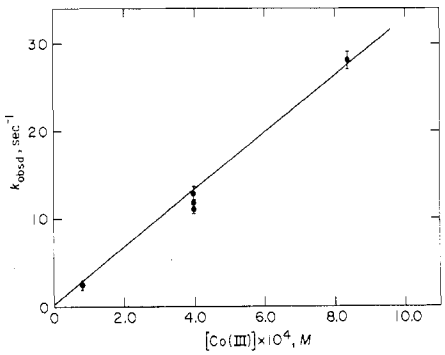

Figure 1. Plot of the observed rate constant vs. $[Co(phen)_3^{3+}]$ for the fast reaction with ferrocytochrome *c* at pH 1.0 (25 °C, μ = 0.13 M (sodium chloride), cytochrome $c = 1 \times 10^{-5}$ M). The reaction was followed at 590 nm.

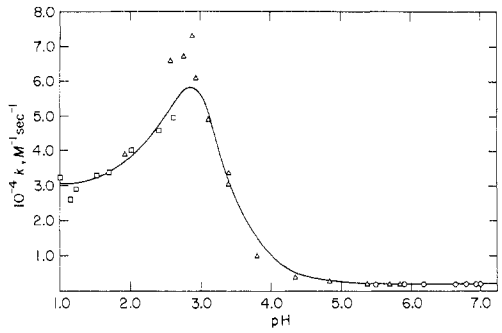

Figure 2. Plot of the second-order rate constant for the electron-transfer reaction between ferrocytochrome *c* and $[Co(phen)_3^{3+}]$ vs. pH (25 °C, μ = 0.13 M (sodium chloride)). The reaction was followed at 590 nm below pH 2.3 and at 550 nm above this pH: ○, phosphate buffer; △, biphthalate buffer; □, no buffer.

are summarized in Table I. A few runs done at pH 7.2 in 2 mM phosphate buffer (μ = 0.10 M, 25 °C) gave rate constants which were in excellent agreement with those previously reported (1.66×10^3 M^{-1} s^{-1}, this work; 1.60×10^3 M^{-1} s^{-1} [11]). In the pH region 5–6 the kinetics were insensitive to a buffer concentration change from 5 to 13 mM. The rates did, however, depend upon the nature of the buffer, being approximately 15% slower in phosphate than in biphthalate.

Below pH 2.3 the rates were wavelength dependent with the kinetic data showing non-first-order behavior at most wavelengths.[15] The reaction under these conditions was studied primarily at 590 and 535 nm. The absorbance changes at the former wavelength gave the most consistently "clean" kinetics while those at the latter wavelength indicated the existence of an intermediate below pH 1.9 (see below). Because the onset of biphasic kinetics at pH ~2.3 is close to the point at which the biphthalate buffer was replaced by hydrochloric acid (pH 2.6), an experiment in which potassium biphthalate was added to the solution was performed at pH 1.92. No change in rate was observed ($(3.9 \pm 0.1) \times 10^4$ M^{-1} s^{-1} with biphthalate vs. $(4.1 \pm 0.1) \times 10^4$ M^{-1} s^{-1} with no biphthalate added).

A distinct slow reaction could be seen below pH 1.9. This slow reaction was studied at 535 nm by either ignoring the initial points or by fitting the reaction curve to a double-exponential decay. The first-order rate constant for the slow reaction determined in this manner was independent of the cobalt(III) concentration from 1.85×10^{-4} to 2.16×10^{-3} M. The rate was only weakly dependent upon pH between 1.0 and 1.7 and could not be observed at and above pH 2.0. The values of the first-order rate constant for the slow reaction are 2.2 ± 0.1, 1.4 ± 0.3, 2.4 ± 0.1, and 2.8 ± 0.5 s^{-1} at pH 1.0, 1.2, 1.5, and 1.7, respectively (μ = 0.13 M, 25 °C). The fast reaction showed a first-order dependence on both the ferrocytochrome *c* and the cobalt(III) concentrations. Figure 1 presents the variation of the pseudo-first-order rate constants with cobalt(III) concentration at pH 1; a least-squares fit of these data gives a rate constant of 3.25×10^4 M^{-1} s^{-1}.

The effect of pH on the second-order rate constants for the oxidation of ferrocytochrome *c* by $Co(phen)_3^{3+}$ is shown in Figure 2. The pH–rate profile goes through a maximum at pH 2.9 ± 0.1 with rate "plateaus" below pH 1.8 and above pH 4.2. This type of behavior has been observed for the chromium(II) reduction of ferricytochrome *c* and was fit by $k = c + d[H^+]$ at high pH and $k = a/[H^+]$ at low pH.[6] In the present work the rate was found to fit the form $k = c + d[H^+]$ at high pH and $k = b + a/[H^+]$ at low pH. The curve drawn in Figure 2 was calculated from

$$k = \frac{b + a/[H^+] + ac/d[H^+]^2}{1 + a/d[H^+]^2} \quad (1)$$

Scheme I

$$\text{ferrocyt } c^1 \rightleftharpoons \text{ferrocyt } c^0 + H^+ \quad K_0'$$
$$\text{ferrocyt } c^2 \rightleftharpoons \text{ferrocyt } c^1 + H^+ \quad K_1'$$
$$\text{ferrocyt } c^2 + \text{Co(III)} \xrightarrow{k_a} \text{products}$$
$$\text{ferrocyt } c^1 + \text{Co(III)} \xrightarrow{k_b} \text{products}$$
$$\text{ferrocyt } c^0 + \text{Co(III)} \xrightarrow{k_c} \text{products}$$

using $a = 80$ s^{-1}, $b = 3.0 \times 10^4$ M^{-1} s^{-1}, $c = 1.8 \times 10^3$ M^{-1} s^{-1}, and $d = 8.0 \times 10^7$ M^{-2} s^{-1}. Equation 1 has the correct limiting forms at high and low pH. The curve fits the kinetic data well above 3.8 and below 2.6. Although the fit is poor in the intermediate pH region, it can be improved at the expense of the fit at the pH extremes or by introducing more terms.

Discussion

The kinetic data show that there is a pH optimum for the rate of oxidation of ferrocytochrome *c* by cobalt(III). A similar type of behavior has been observed in the reduction of ferricytochrome by chromium(II) and by $Ru(NH_3)_6^{2+}$.[6,16] These reactions were interpreted in terms of acid-dependent equilibria between three forms of ferricytochrome, with each form having a different reduction rate. The present data can also be interpreted in this manner (Scheme I).

In this scheme ferrocyt c^0 is the native form of the protein (stable at pH 6) while ferrocyt c^1 and ferrocyt c^2 have added one and two protons, respectively. The above scheme leads to the following expression for the second-order rate constant provided the acid-dependent equilibria are established rapidly relative to the redox reactions. Equation 2 is identical with

$$k = \frac{k_a + k_b K_1'/[H^+] + k_c K_1' K_0'/[H^+]^2}{1 + K_1'/[H^+] + K_1' K_0'/[H^+]^2} \quad (2)$$

eq 1 if the second term in the denominator of eq 2 is neglected. In terms of this interpretation, $k_a = 3.0 \times 10^4$ M^{-1} s^{-1}, $K_1' < 1 \times 10^{-3}$ M, $k_b > 8 \times 10^4$ M^{-1} s^{-1}, $K_0' > 1 \times 10^{-3}$ M, $k_c = 1.8 \times 10^3$ M^{-1} s^{-1}, and $K_1'K_0' = 1 \times 10^{-6}$ M^2. The singly protonated species (ferrocyt c^1), while never significant in concentration, is the most rapidly oxidized form and is responsible for the rate maximum at pH 2.9. This behavior is a consequence of $K_0' > K_1'$ while $k_b > k_a$ and $k_b > k_c$.

Although the nature of the protonated forms of ferrocytochrome *c* is not known, there is good evidence that the iron–protein bonds are broken at low pH. Thus ferrocytochrome *c* begins to bind carbon monoxide at pH 3[17–20] indicating that the protein is at least partially denatured. In neutral solution ferrocytochrome *c* is unaffected by oxygen and is stable over a period of hours. However, it is rapidly oxidized by air either when denaturing agents are added to the solution[20] or when the pH is lowered below 3.[21] The susceptibility to oxidation by O_2 is usually associated with a

Scheme II

$$\text{ferrocyt } c^2 + \text{Co(III)} \xrightarrow{k_1} \text{ferricyt } c^2 + \text{Co(II)}$$

$$\text{ferricyt } c^2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{ferricyt } c^3$$

coordination site being available to bind the O_2 to the iron(II).[20] All of the above imply that one or both of iron–protein bonds (either the bond to methionine-80 or to histidine-18) is broken at pH <3. McDonald and Phillips[22] have found that the ^1H NMR spectra of the ferroprotein starts to change significantly at pH 4.5 and is greatly changed by pH 3; the resonances from both the Met-80 and His-18 have disappeared by the latter pH. They concluded that either these ligands are no longer bound to the iron(II) or the iron(II) has become high spin and severely broadened their resonances.[22] Despite the above evidence for substantial changes in the ferrocytochrome *c* as the pH is lowered, no changes were observed in the visible spectrum in the pH range 1–6.

This discussion has assumed that the variation in rate with pH is due primarily to changes undergone by the ferrocytochrome *c*. No consideration has been given to changes in the $Co(phen)_3^{3+}$. This seems reasonable since the cobalt(III) complex does not manifest a p*K* in the pH range 1–7. This interpretation is further supported by the fact that a similar pH–rate profile has been found for the reduction of ferricytochrome by chromium(II)[6] and $Ru(NH_3)_6^{2+}$.[16] The ferricytochrome *c* catalyzed oxidation of pyrogallol to purpurogallin by hydrogen peroxide has a maximum in its pH–rate profile at pH 3.5.[23] Also the oxidation of ferrocytochrome by alkyl halides increases in rate as the pH is lowered.[24] Unfortunately in the latter study the change in rate with pH was measured at only two values below pH 4 and it is unclear whether there is a rate maximum between pH 2 and 3.

The similarity in the pH for the rate maximum, independent of the oxidation state of the iron, makes it attractive to postulate that the protonation site is remote from the iron. If the protein undergoes protonation at a pH that is independent of the iron oxidation state, then the interaction between the iron and the site of protonation must be weak. Cohen and Hayes reported that for the oxidized protein the histidine-26 becomes protonated at pH 3.5.[25] The histidine is believed to be hydrogen bonded to the peptide bond carbonyl oxygen atom of proline-44.[2] Cohen and Hayes speculated that the breaking of this hydrogen bond could be one of the first steps in the acid-induced denaturation of the protein.[25] If the above is true, then the denaturation of the reduced protein might also begin by this protonation step at a very similar pH. Therefore, the peak in the pH–rate curve would be expected to be in a similar position for both the oxidation and the reduction of the protein, as is in fact observed.

Below pH 2 biphasic kinetics are observed, the second reaction being both much slower than the redox reaction and independent of oxidant concentration. This second reaction is identified with a conformation change of the protein. Such a change can occur either before or after the electron transfer and there is ample evidence for both types of change. Thus a preredox conformation change has been considered in the oxidation of ferrocytochrome *c* by substituted ferricyanides[26] and in the reduction of ferricytochrome *c* by chromium(II) at low pH.[27] On the other hand, the oxidation of ferrocytochrome *c* by $Fe(CN)_6^{3-}$ at high pH has been interpreted in terms of postredox conformation changes in the iron(III) protein.[28] In the reduction of cyanoferricytochrome *c* by dithionite an initial electron-transfer reaction is followed by a conformation change in which the cyanide is eliminated from the iron(II) protein.[29] Finally, studies of the oxidation of ferrocytochrome *c* by $Co(phen)_3^{3+}$ at high pH have shown that an unstable conformation of ferricytochrome *c* is produced in this reaction.[11]

The results of the present study are consistent with a postredox mechanism involving the formation of an unstable ferricytochrome *c* species. The preredox mechanism is inconsistent with the kinetics and the observed spectral changes.[30] For the present system Scheme II is therefore postulated. We next consider the nature of ferricyt c^2, the unstable ferricytochrome *c* species produced in the redox reaction below pH 1.7. There is good evidence that this species is the mixed-spin form of the iron(III) protein. The native iron(III) protein is low spin while at pH <3 a high-spin form (five unpaired electrons) is also found.[31] At high ionic strength and pH <2 a mixed-spin iron(III) with three unpaired electrons is observed.[32,33] The rate of conversion of the high-spin to the mixed-spin form has been measured by Aviram, who reported a rate constant of $\sim 5\ s^{-1}$ for the interconversion at pH 1.8 (μ = 0.15 M (sodium chloride)).[33] We have measured, by an ionic strength jump on the stopped-flow spectrophotometer, rates of $2\ s^{-1}$ at pH 1 and $8\ s^{-1}$ at pH 2 (μ = 0.13 M (sodium chloride)). The observed rate constant for the interconversion is equal to the sum of the forward and reverse rate constants regardless of which form was present initially. Therefore, if ferricyt c^2 is identified with the mixed-spin form and ferricyt c^3 with the high-spin form, then the rate observed in the ionic strength jump (ferricyt c^3 initially) should be the same as that observed for the slow reaction in the redox reaction (ferricyt c^2 initially).[34] This is indeed the case ($2\ s^{-1}$ vs. $2.2 \pm 0.1\ s^{-1}$ at pH 1). At pH 2 the slow conversion of ferricyt c^2 to ferricyt c^3 is no longer seen in the redox reaction because the mixed-spin form is the dominant form at this pH. Finally, the absorbance changes expected for this interpretation can be calculated from the known spectra of the mixed-spin and high-spin forms of the ferricytochrome *c*. The changes in absorbance observed in the slow reaction are in good agreement with these predictions.

To summarize, the present investigation shows that the mixed-spin form of ferricytochrome *c* is the primary product of the oxidation of ferrocytochrome *c* by $Co(phen)_3^{3+}$ at pH $\sim$1. This conclusion is consistent with the result of studies of the reduction of ferricytochrome *c* by chromium(II) at low pH[27] and with kinetic studies of the interconversion of the various forms of ferricytochrome *c*.[33] It will be of interest to examine the relative reactivities of these forms in other reactions of cytochrome *c*.

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Registry No. Ferrocytochrome *c*, 9007-43-6; tris(1,10-phenanthroline)cobalt(III), 18581-79-8.

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Contribution from the Lash Miller Chemical Laboratories, University of Toronto, Toronto, Ontario, M5S 1A1, Canada

Dissymmetric Arsine Complexes. Cobalt Hydrides and Cobalt Dioxygen Complexes

B. BOSNICH,* HEATHER BOUCHER, and CEDRIC MARSHALL

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Cobalt hydrido and "sideways" bonded dioxygen complexes have been prepared. The complexes contain two linear quadridentate arsine ligands, one of which is flexible in the topologies it can adopt while the other forces a cis-α topology of the complexes. It is found that the protic and hydridic character of the hydrido ligands is modified by the imposition of a cis-α topology. The "sideways" bonded dioxygen complexes are best described as cobalt(III) peroxide species and their stability is essentially unaffected when the coordination angle trans to the dioxygen moiety is less than 90°.

Conventional cobalt complexes, namely, those with "hard" donor ligands, are decomposed to the metal in the presence of hydride ions, and the dioxygen complexes, which are usually derived from the divalent oxidation state, invariably give either the μ-peroxo dimers or superoxo monomers.[1,2] With "soft" donor atoms, such as phosphorus or arsenic, or with the cyanide ligand, by contrast, hydride ion reduction generally produces hydrido complexes of varying stability[3-8] and the now-accessible cobalt(I) state reacts with oxygen to give "sideways" bonded dioxygen complexes of remarkable stability.[9,10] It is evident from the high intensities of the d–d transitions and the drastically reduced (free-ion) interelectronic repulsions,[11-13] that the d orbitals of the metal have become more diffuse and polarizable when the metal is surrounded by soft ligands, and it has been suggested[8,13] that it is this property which gives rise to the stability of the hydrides, the "sideways" bonded dioxygen species, the accessibility of the cobalt(I) state, and the consequent organometallic reactivity. Given that these electronic conditions are fulfilled, there remains an intriguing stereochemical feature which may determine the reactivity of the hydrides and the stability of the dioxygen complexes.

Both of the "sideways" bonded dioxygen–cobalt complexes which have had their (crystal) structures determined, one with phosphine[9] and the other with arsine ligands,[14] are grossly distorted octahedra. In particular, the donor atom–cobalt angle trans to the cobalt–dioxygen plane is large (~110°) (see Figure 4) and similar to the corresponding angles observed for rhodium and iridium dioxygen complexes.[15,16] This has led to the inference that the bonding and oxidation state of these species may be unusual and that the bonding may be described by schemes reminiscent of the Chatt–Dewar model for ethylene binding.

The other stereochemical aspect relates to the dichotomous chemical reactivity of dihydrocobalt complexes, where it is observed for the *cis*-$[Co(diars)_2(H)_2]^+$ ion (diars = *o*-phenylenebis(dimethylarsine)) that, after the first hydrido ligand is induced to react as a hydride, the trans monohydrido species so formed displays no hydridic character under normal conditions.[8] The second hydrido ligand reacts as a proton. This probably also obtains for the $(diphos)_2$ analogue of Sacco,[5] although it has not been investigated in the same detail. The question then arises as to whether this dichotomous reactivity is a consequence of the cis to trans rearrangement which occurs after the release of the first hydride.

This paper describes some of the hydrido and dioxygen complexes of cobalt associated with two linear quadridentate arsine ligands, one of which is flexible in the topologies it can adopt and the other is rigid and forces a cis arrangement of the two remaining octahedral positions, while, at the same time, the arsenic–cobalt–arsenic angle trans to the two substitutional sites cannot expand beyond 90°.

1. Stereochemistry

The structures of the two arsines used are shown in Figure 1, and it will be noted that both have racemic configurations of the inner arsenic atoms, which are geometrically stable under the present experimental conditions. We have shown elsewhere[17] that the racemic fars ligand can adopt any of the cis-α, cis-β, and trans topologies about an octahedral metal center, and there appears to be no strong preference for any one of these. The racemic qars ligand, by contrast, is inflexible and only adopts the symmetrical cis-α geometry.[17] Indeed, so strong does this preference seem to be, that the crystal structure of $[Pd(R,R:S,S\text{-qars})Cl]ClO_4$ resembles a cis-α octahedral complex with a vacant site.[18] Moreover, the inner arsenic–palladium–arsenic angle is 82°.

2. Hydrides

When the deep green *cis*-α-$[Co(R,R:S,S\text{-fars})Br_2]Br$ complex is reduced with BH_4^- ions in neutral aqueous methanol, a transient straw yellow solution is produced which rapidly fades in the presence of oxygen to produce the deep brown "sideways" bonded dioxygen adduct *cis*-β-$[Co(R,R:S,S\text{-fars})O_2]^+$. The straw yellow solution contains *trans*-$[Co(R,R:S,S\text{-fars})(H)Br]^+$ which, under the basic reaction conditions, is deprotonated to give a Co(I) species which reacts with oxygen. If, however, the reduction is carried out under acidic conditions, with acetic acid, the deprotonation is suppressed and the yellow *trans*-$[Co(R,R:S,S\text{-fars})(H)Br]ClO_4$ complex can be isolated. In acidic or neutral solutions