Indirect Determination of the Rate of Iron Uptake into the Apoprotein of the Ribonucleotide Reductase of *E. coli*†

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ABSTRACT: The second-order rate constant k_{apo} for uptake of Fe^{II} by the apoprotein of Ribonucleotide Reductase R2 has been measured by letting that reaction compete with the uptake of Fe^{II} by ferrozine (rate constant k_{Fz}). The rate of the Fe^{II}/ferrozine reaction was studied at high ferrozine concentrations, and an effective first-order rate constant k_{Fz} for the disappearance of Fe^{II} determined in the presence of bovine serum albumin as a viscogen. Solutions of apoprotein and ferrozine in various ratios were mixed with Fe^{II} solutions in a stopped-flow apparatus, and the growth of the 562 nm Fe^{II}(ferrozine)$_3$ absorbance monitored. Attempts to fit the data to a variety of kinetic schemes imply that uptake of the second Fe^{II} by apo is slower than uptake of the first, suggesting that the rate-determining step in the activation of R2 is a conformational change *after* the uptake of the first iron. The resulting value of k_{apo} is $1.8(1) \times 10^6$ M^{-1} s^{-1}.

In its active form the R2 subunit of *Escherichia coli* ribonucleotide reductase contains a μ-oxo diiron(III) cluster and a stable tyrosyl radical (•Y122) essential for activity (*1*−*8*). These components can only be generated by the reaction of O_2 with protein that contains Fe(II). If the •Y122 of active R2 becomes reduced, and the activity of the protein thereby quenched, the μ-oxo diiron(III) cluster must be reduced back to the Fe(II) stage before the Y122 can be reoxidized (*9*).

Previous studies of the activation of R2 have identified an intermediate **X**, which appears to be a spin-coupled $(H_2O)Fe^{III}OFe^{IV}$ fragment (*10*, *11*); **X** is converted to active R2 at a rate of 1 s^{-1}. As long as Fe^{2+} is present in excess (or if ascorbate is present as a one-electron reducing agent), **X** is formed from apoprotein, Fe^{2+}, and O_2 at a rate first-order in apoprotein (approximately 10 s^{-1}) but *independent* of the concentrations of Fe^{2+} and of O_2 (*12*, *13*). The rate-determining step in the formation of **X** thus does not involve either Fe^{2+} or O_2. Stubbe has suggested that a conformational change must occur in the apoprotein before the Fe^{2+} is incorporated (*14*). Her group has shown that apoprotein preloaded with 2 equiv of Fe(II) (diferrous R2) reacts with oxygen (eq 1) an order of magnitude faster (60−80 s^{-1}) than apoprotein freshly mixed with aq Fe(II); Bollinger and co-workers have found the second-order rate constant at 5 °C to be 2.2×10^5 M^{-1} s^{-1} (*15*).

Thus the rate-determining step in the activation of R2 must occur *before* the *second* Fe(II) is incorporated. Stubbe has remarked that "The kinetics suggest that formation of the diferrous-R2 complex from apo[1] is slow" (*14*). However, neither of the above results excludes a rate-determining conformational change that occurs (eq 3) *between* eq 2 and eq 4 (*16*).

$$\text{apoFe}_2 \xrightarrow{O_2,\text{fast}} \text{X (60-80 s}^{-1}\text{), then to active R2 (1 s}^{-1}\text{)} \quad \text{(1, observed)}$$

$$\text{apo} + \text{Fe}^{II} \xrightarrow{k_{apo}} \text{apoFe} \quad \text{(2, proposed)}$$

$$\text{apoFe} \xrightarrow{k_{conf}} \text{(apo′)Fe} \quad \text{(3, proposed)}$$

$$\text{(apo′)Fe} + \text{Fe}^{II} \xrightarrow{k_{apoFe},\text{fast}} \text{apoFe}_2 \quad \text{(4, proposed)}$$

This hypothesis creates the need to determine the kinetics of Fe(II) uptake by the R2 apoprotein. The rate of the first Fe(II) uptake will be independent of Fe(II) if it is preceded by a rate-determining conformational change, and first order in Fe(II) if it occurs by eq 2.

Unfortunately, Fe^{2+} at micromolar concentrations is spectroscopically invisible (except in Mössbauer, by which kinetics are cumbersome). UV−vis spectroscopy cannot be used to monitor the uptake of Fe(II) by the apoprotein of R2. We have therefore determined the kinetics of Fe(II) uptake *indirectly*, by letting it compete with the spectroscopically observable reaction between Fe(II) and ferrozine. [The deep magenta (ferrozine)$_3Fe^{II}$ complex has $\epsilon_{562nm} = 27\,900$ M^{-1} cm^{-1} (*17*).]

Previous Kinetic Studies on Chelation of Fe(II) by Ferrozine. In 1984, Thompsen and Mottola, using 800 μM

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[1] Abbreviations: apo, the metal-free form of *E. coli* ribonucleotide reductase R2; apo′, apo before iron uptake but after a conformational change; apoFe, apo with one bound Fe^{2+} prior to any conformational change; (apo′)Fe, apo with one bound Fe^{2+} after a conformational change; apoFe2, R2 with two bound Fe^{2+} (reduced R2); Fz, ferrozine; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid, monosodium salt; BSA, bovine serum albumin.

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ferrozine, an ionic strength of 0.1 M, and a pH range of 2.8−5.5, reported an average ferrozine reaction order of 2.95 and a forward rate constant of 3.08(3) × 10^{11} M^{-3} s^{-1} at 20 °C (*18*). They therefore concluded that the reaction was first order in Fe^{2+} and third order in ferrozine (fourth order overall) (eq 8) and proposed a mechanism consisting of the three steps in eqs 5−7.

$$Fe^{II} + Fz \overset{K_1}{\rightleftharpoons} FeFz \quad (5)$$

$$FeFz + Fz \overset{K_2}{\rightleftharpoons} FeFz_2 \quad (6)$$

$$FeFz_2 + Fz \xrightarrow{k_3} FeFz_3 \quad (7)$$

$$\frac{d[FeFz_3]}{dt} = K_1K_2k_3[Fe(II)][Fz]^3 = k_{Fz}[Fe^{II}] \quad (8)$$

$$k_{Fz} = K_1K_2k_3[Fz]^3 \quad (9)$$

Independently in 1992 another group studied from 15 to 32 °C the kinetics of the ferrozine-Fe^{2+} reaction at 28−80 μM ferrozine (an order of magnitude lower than the concentration Mottola employed), salinity 0−36%, and pH 6.0−8.4; they found the order in ferrozine to be 2.91(4) and the rate constant to be about 2.0 × 10^{11} M^{-3} s^{-1} (*19*). Considering the differences in pH, ionic strength and temperature between these two studies, the agreement is remarkable.

EXPERIMENTAL PROCEDURES

Materials. The *E. coli* ribonucleotide reductase R2 was produced in the overproducing strain BL21DE3 (Novagen) containing the plasmid pR2wt-*Hind*III, and isolated in the apo form as previously reported (*20*). Bovine serum albumin (Cohn V fractionate powder), HEPES, sodium ascorbate, ferrous ammonium sulfate hexahydrate, and ferrozine were purchased from Sigma-Aldrich and used as received. Type I reagent grade water (Barnstead/Thermolyne model D4751) was used for all solutions.

HEPES buffer was made up as 100 mM (to account for the 2-fold dilution in the stopped-flow apparatus) from the free acid form. The solution was chilled to 4−5 °C, then titrated to pH 7.6 using NaOH, and the solution made to volume.

Methods. Kinetic data were collected over the range of approximately 390−610 nm with an SF-40 Canterbury Stopped-Flow (Hi-Tech Scientific) thermostated to 5.0 (1) °C, with an RSM-1000 rapid-scanning device (On-Line Instrument Systems) and software. Scans were collected in dual beam mode, 1000 scans/s. Total reaction volume per "shot" was approximately 200 μL. Stopped-flow data were fit iteratively using the kinetic modeling and simulation program MacKinetics (developed by Walter S. Leopold III, formerly of E. I. du Pont de Nemours, Inc.).

Kinetics of Fe^{2+}/Ferrozine in HEPES Buffer. (Unless otherwise noted, all concentrations listed are those placed in the syringes, i.e., before mixing in the stopped-flow apparatus.) Ferrozine (686 μM to 4.37 mM) was dissolved in 100 mM HEPES buffer; ferrous ammonium sulfate (60 μM) was dissolved in 5 mN H_2SO_4 with enough NaCl added to give a final (postmixing) ionic strength of 0.1 M. Fresh ferrozine and iron solutions were made each day; all reactions were done in air.

BSA as a Viscogen in Fe^{2+}/Ferrozine. A 400 μM solution of BSA was prepared from a solution of ferrozine (3.4 mM) in HEPES buffer; dilution with more Fz/HEPES solution provided BSA concentrations from 25 to 350 μM. Fresh iron/salt solution was made as above, and the ferrozine-iron reactions carried out in the stopped-flow apparatus at 5.0 °C.

Competition between Fz and Apo for Iron(II). Ferrozine (1.4−8.7 mM), apoR2 (24−198 μM), and 3 mM sodium ascorbate were dissolved in HEPES buffer and loaded into one syringe of the stopped-flow apparatus. Fresh iron/salt solution, made as in the previous sections, was placed in the other syringe.

RESULTS

Preliminary Stopped-Flow Experiments Showed Competition between Apoprotein and Ferrozine for Fe(II). Solutions containing appropriate amounts of ferrozine (Fz), apoprotein, and sodium ascorbate, buffered with HEPES at pH 7.6, were mixed at 5.0 °C with equal volumes of Fe^{2+} solutions; the resulting mixtures were 33.0 μM in Fe^{2+}. The absorbance due to the interaction of Fe^{II} with ascorbate was negligible at the iron concentrations employed.

The experiments were done in air so that any $apoFe_2$ formed would be converted to active R2; the ascorbate guaranteed that **X** was the first observable intermediate in the $apoFe_2/O_2$ reaction and that it was the source of all of the resulting •Y122.[2] The experiment was repeated with various [ferrozine]/[apoprotein] ratios, and the amount of $Fe^{II}Fz_3$ formed in each case was determined from the absorbance at 562 nm.

At [ferrozine]/[apoprotein] ratios of >100, ferrozine complexed all the Fe(II), whereas at [ferrozine]/[apoprotein] ratios of <8, ferrozine did not disturb the uptake of Fe(II) by the protein. Detectable amounts of •Y122 were formed from apoprotein only if [ferrozine] was lowered to a near-stoichiometric ratio with iron (just 3.2 Fz/Fe). Good competition, with both apoprotein and ferrozine trapping some iron, was observed at an [Fz]/[Fe]/[apoprotein] ratio of 45:2.3:1.

The order in ferrozine of its coordination by Fe(II) proved to decrease at the higher [Fz] necessary for competition with apoprotein. Equations 8 and 9 are correct *only* if both the K_1 and K_2 equilibria (eqs 5 and 6) lie to the left, a requirement that will cease to be met at sufficiently high [Fz]. An appreciable fraction of the total iron, T (eq 10), will then be FeFz and $FeFz_2$ after mixing.

$$T = [Fe^{2+}] + [FeFz] + [FeFz_2] \quad (10)$$

Substitution of expressions for K_1 and K_2 into eq 10 gives (eqs 11 and 12) the equilibrium expression for $[FeFz_2]$. Consideration of eq 7 leads to the complete rate law in eqs

[2] The cation radical •W48H$^+$ (which absorbs at 560 nm) is initially formed along with **X** from the reaction of Fe^{2+}, apoR2, and oxygen (*12*, *13*, *15*). Either ascorbate or excess Fe^{2+} rapidly reduces the •W48H$^+$, leaving **X** to oxidize Y122 to •Y122. Only ascorbate was practical in our competition experiments, as they required that iron be the limiting reagent.

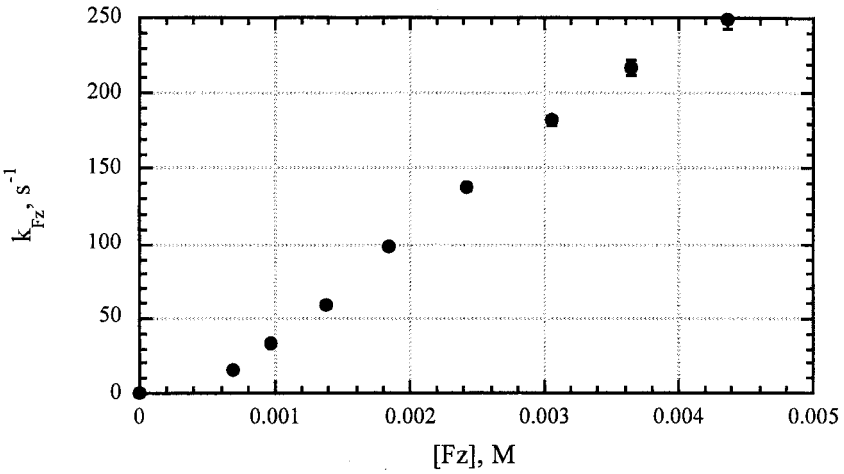

FIGURE 1: Plot of data from Table 1. Conditions: 50 mM HEPES, pH 7.6 at 5.0 °C; 30 μM iron(II), $I = 0.1$ M.

13 and 14. The limiting order in ferrozine should be three at low [Fz] and one at high [Fz].

$$T = \frac{[FeFz_2]}{K_1K_2[Fz]^2} + \frac{[FeFz_2]}{K_2[Fz]} + [FeFz_2] \qquad (11)$$

$$[FeFz_2] = \frac{TK_1K_2[Fz]^2}{1 + K_1[Fz] + K_1K_2[Fz]^2} \qquad (12)$$

$$\frac{d[FeFz_3]}{dt} = \text{rate} = \frac{K_1K_2k_3[Fz]^3T}{1 + K_1[Fz] + K_1K_2[Fz]^2} = k_{Fz}T \qquad (13)$$

$$k_{Fz} = \frac{K_1K_2k_3[Fz]^3}{1 + K_1[Fz] + K_1K_2[Fz]^2} \qquad (14)$$

We have redetermined the kinetics of the ferrozine reaction at higher concentrations than those employed by previous workers [28−80 μM, Lin and Kester (*19*); 800 μM, Mottola (*18*)], under conditions appropriate for the uptake of Fe(II) by apoprotein (5.0 °C, pH 7.6 with HEPES as a buffer, and $I = 0.1$ M). The resulting values of the pseudo-first-order rate constant k_{Fz} as a function of [Fz] are given in Table S-1 and shown in Figure 1; a plot of $\ln(k_{Fz})$ vs ln[Fz] (Figure S-1) implies an order of 1.14 at [Fz] > 2.4 mM, and an order of 2.16 at [Fz] < 1.9 mM. Higher [Fz] were impractical because the rates became too fast to measure.

From these data we can infer values for k_3 and the product K_1K_2. If we assume that the reaction is first order at [Fz] = 4.37 mM, eq 14 reduces to $k_{Fz} = k_3[Fz]$; the experimental k_{Fz} gives an estimate for k_3 of 5.8×10^4 M^{-1} s^{-1}. At [Fz] = 686 μM, low enough for the reaction to be third order, eq 14 simplifies to $k_{Fz} = K_1K_2k_3[Fz]^3$; the experimental k_{Fz} and our estimate for k_3 give an estimate of 1.32×10^6 M^{-2} for K_1K_2. Fitting the data (Table S-1 in Supporting Information and Figure 1) to eq 14, with K_1K_2 constrained to be 1.32×10^6 M^{-2}, suggests that $k_3 \approx 7.0 \times 10^4$ M^{-1} s^{-1}.

The binding constant for the third ferrozine is undoubtedly larger than for the first two because of the change in the spin of the Fe^{II}, as observed classically (*21, 22*) for Fe(*o*-phenanthroline)$_3^{2+}$ and Fe(2,2′-bipyridine)$_3^{2+}$. For the same reason the third equilbrium is established more slowly than the first two.

Viscosity Effects on the Rate of Iron Uptake by Ferrozine. During our preliminary studies of ferrozine/apoprotein competition for Fe^{2+}, we determined approximate first-order rate constants k_{obs} by monitoring the growth of the $Fe^{II}Fz_3$ absorbance at 562 nm. Because k_{obs} should be the sum of k_{Fz} and the first-order rate constant for uptake of Fe^{2+} by apoprotein, we expected an increase in k_{obs} when [apoprotein] was increased at constant [Fz]. While the outcome of the reaction—the fraction of the initial Fe^{2+} converted to $Fe^{II}Fz_3$—was plainly the result of competition between ferrozine and apoprotein, k_{obs} actually *decreased* (Figure S-2 in Supporting Information).

Because a high concentration of protein (up to 146 μM) had been required in order to compete with ferrozine for the Fe^{2+} and that concentration had plainly increased the viscosity of the solution, we explored the effect of viscosity on k_{Fz}. Bovine serum albumin (BSA) proved ideal as a "proteinic viscogen" (*23*) to mimic the effects of R2. BSA is available in high purity, stable over a wide pH range, and very soluble even at 5 °C, and has a molecular weight of 66.7K (*24*), close to that of R2 (87K) (*25*). One BSA conformation, the "N form," is even heart-shaped (*26*), somewhat like R2 (*27*).

BSA does not bind Fe^{2+} strongly; a recent paper notes that "the affinity of free iron for albumin is rather weak" (*28*). Even that weak interaction, occasionally suggested in the literature (*29, 30*), may be due to partial oxidation of the iron during the relevant experiments. [Fe^{II} is rapidly oxidized to Fe^{III} in phosphate buffer (*25*) and Fe^{III} binds strongly to BSA (*31*−*33*).] However, as serum albumins are known to bind Co^{2+} weakly (*25, 34*), we tested the effect of BSA on the uptake of Fe^{II} by ferrozine; the formation of $Fe^{II}Fz_3$ remained almost quantitative (an average of 98% of that expected from the Fe^{2+} initially present) (Figure S-3 in Supporting Information). Experiments run at very high [BSA], for instance 200 μM, only lost 1−2 μM (3−6%) of the total Fe^{II} in solution.

Figure 2 shows how BSA affected the uptake of Fe^{II} by Fz: the absorbance due to $Fe^{II}Fz_3$ grew more slowly with increasing BSA, but approached the same final value. The decrease of the measured k_{Fz} with BSA is shown in Figure 3. A similar inverse dependence on viscosity has been noted

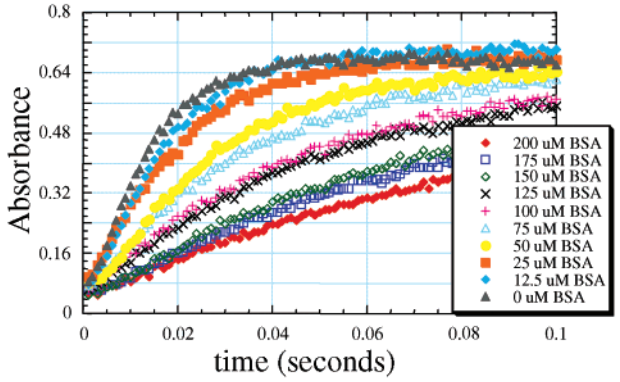

FIGURE 2: Effect of viscosity from added BSA on the ferrozine + Fe(II) reaction. Conditions after mixing in the stopped-flow: I = 0.1 M (NaCl), 5.0 °C, pH 7.6, with HEPES 50 mM; Fz = 1.7 mM; Fe(II) = 30 μM; BSA 0−200 μM.

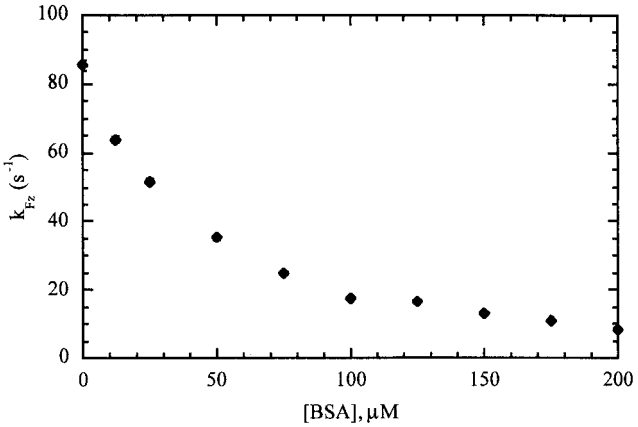

FIGURE 3: Observed rate constants for the ferrozine + Fe(II) reaction with BSA present. Postmixing conditions I= 0.1 M (NaCl), 5.0 °C, pH 7.6 with HEPES 50 mM; Fz = 1.7 mM; Fe(II) = 30 μM; BSA = 0−200 μM.

for the reactions of small ligands (O_2 and CO) with the heme protein myoglobin (*35*) and for the reactions of various catalases with their substrate H_2O_2 (*36*).

The k_{Fz} values used in evaluating the apo/Fz competition experiments were therefore measured with BSA present as a viscogen. The amount of BSA added during a given k_{Fz} measurement equaled (same mg/mL) the amount of apo to be used in the corresponding apo/Fz competition experiment; the other conditions (I = 0.1 M, pH 7.6 with 50 mM HEPES buffer, 5.0 °C) were the same as those to be used in the competition experiments. The results are included in Table 1 below.

Quantitative Assessment of Competition between Apoprotein and Ferrozine for Fe(II). Again, the experiments were done in air so that any $apoFe_2$ formed would be converted to active R2, but [apo] was kept greater than [Fe^{II}] in order to simplify kinetic modeling; under these conditions little $apoFe_2$ and thus little •Y122 were formed. One syringe of the stopped-flow apparatus contained a freshly prepared 60 μM solution of Fe^{2+}, along with enough NaCl to give a final (i.e., after mixing) ionic strength of 0.1 M. The other syringe contained appropriate concentrations of ferrozine and apoR2, along with 3 mM sodium ascorbate, in 100 mM HEPES buffer (pH 7.6). Ferrozine was used in excess sufficient to establish pseudo-first-order conditions and to ensure that the rate of formation of $Fe^{II}Fz_3$ was always $k_{Fz}T$ (see eqs 11 and 13). It was not practical to use a large excess of apoprotein (too much protein would have been required, and the viscosity effects would have been too great). The details of each experiment are listed in Table 2.

Table 1: Rate Constants in Ferrozine/Apoprotein Competition Experiments[a]

protein (μM)	Fz (mM)	k_{Fz} (s^{-1})	$10^6 \times k_{apo}$ (M^{-1} s^{-1})	% of $[Fe]_o$ as $FeFz_3$
31.1 (BSA)	1.83	55.5		99
24.4 (apo)	1.83	55.5	3.38	38
38.1 (BSA)	2.01	42.0		89
29.3 (apo)	2.01	42.0	2.12	37
191.0 (BSA)	1.23	5.27		103
146.4 (apo)	1.23	5.27	0.7	8
127.3 (BSA)	2.07	34.7		100
97.6 (apo)	2.07	34.7	2.56	16
127.3 (BSA)	2.53	34.5		95
97.6 (apo)	2.53	34.5	1.58	18
127.3 (BSA)	3.80	78.6		90
97.6 (apo)	3.80	78.6	1.71	38
214.8 (BSA)	1.83	38.7		94
164.7 (apo)	1.83	38.7	0.8	19
257.0 (BSA)	2.01	39.0		93
197.6 (apo)	2.01	39.0	0.8	20
126.9 (BSA)	1.32	31.7		98
97.3 (apo)	1.32	31.7	1.14	18
126.9 (BSA)	1.98	58.2		98
97.3 (apo)	1.98	58.2	1.14	25

[a] For each ferrozine concentration the first line reports the apparent first-order rate constant, k_{Fz}, in the presence of BSA as a viscogen; the second line reports the value of k_{apo} that best fits (by iteration with MacKinetics) $[FeFz_3]_t$ data obtained in the presence of the same weight of apo. The last column is the percentage of $[Fe]_0$ ultimately trapped by ferrozine.

Modeling. The fact that apoprotein was not present in large excess meant that uptake of Fe^{II} by apoprotein could not be treated as a pseudo-first-order process in competition with the pseudo-first-order rate constant k_{Fz}. The situation was, however, easily modeled by a kinetic simulation program (MacKinetics).

If we consider a second-order rate law, with rate constant k_{apo}, for the uptake of the first Fe^{II} by apoprotein (eq 15),[3] and let it compete for Fe^{II} with the known k_{Fz} (eq 16), it is possible to determine k_{apo} from the time dependence of $[FeFz_3]$.

$$\text{apo} + Fe^{II} \rightarrow \text{apoFe}, k_{apo} \quad (15)$$

$$Fe^{II} \rightarrow FeFz_3, k_{Fz} \quad (16)$$

To have our simulations calculate the amount of ferrozine consumed by eq 16 while retaining k_{Fz} as a pseudo-first-order rate constant, we used the artificial sequence in eqs 17 and 18. The first (eq 17) shows the first-order (rate constant k_{Fz}) conversion of Fe^{II} to the imaginary species "Q", while the second (eq 18) calculates the amount of ferrozine consumed in a fast but formally separate reaction as Q becomes $FeFz_3$. The results (an example of which is shown

[3] The "Fe^{II}" in eq 15 is technically T from eq 10, the total of [Fe^{2+}], [FeFz], and [$FeFz_2$]. It might thus vary with [Fz], but (because we have been unable to determine K_2 independently of K_1) we cannot predict the amount of variance with [Fz]. The fact that the k_{apo} values in Table 2 do not vary systematically with [Fz] implies that k_{obs} is little affected by the position of the equilibria in eqs 5 and 6. The value of k_{obs} for free [Fe^{2+}] may differ somewhat from our result.

Table 2: Rate Constants in Ferrozine/Apoprotein Competition Experiments from a Model Including a Conformational Change (eq 20) at 10 s^{-1}[a]

run	[apo] (μM)	[Fz] (mM)	k_{Fz} (s^{-1})	% yield of apoFe	% yield of $FeFz_3$	$[FeFz_3]_{final}$ (μM)	$[FeFz_3]_0$ (μM)	$10^6 \times k_{apo}$ (M^{-1} s^{-1})
1	24.4	1.83	55.5	62	38.0	11.4	2.2	*b*
2	29.3	2.01	42.0	63	37.1	11.0	2.0	*b*
3	146.4	1.23	5.27	92	8.3	2.5	1.6	*c*
4	97.6	2.07	34.7	84	16.3	4.6	2.5	1.88
5	97.6	2.53	34.5	82	17.7	5.5	2.0	1.70
6	97.6	3.80	78.6	62	37.6	9.0	3.0	2.18
7	164.7	1.83	38.7	81.3	18.7	5.7	1.5	1.18
8	197.6	2.01	39.0	80	19.6	6.1	2.5	1.55
9	97.3	1.32	31.7	82.5	17.5	5.75	1.5	1.83
10	97.3	1.98	58.2	75.4	24.6	8.0	0.5	2.28
								1.80(14) $\times$ 10^6

[a] For each [apo]/[Fz] combination k_{Fz} is the apparent first-order rate constant in the presence of BSA as a viscogen; the weight of BSA in these experiments is equal to that of the apo it replaces. The values of k_{apo} are those that best fit the $[FeFz_3]_t$ data. [b] No value given because it was impractical to correct for the dead time of the stopped-flow apparatus (see text). [c] No value given because too little $FeFz_3$ was formed for its concentration to be measured accurately (see text).

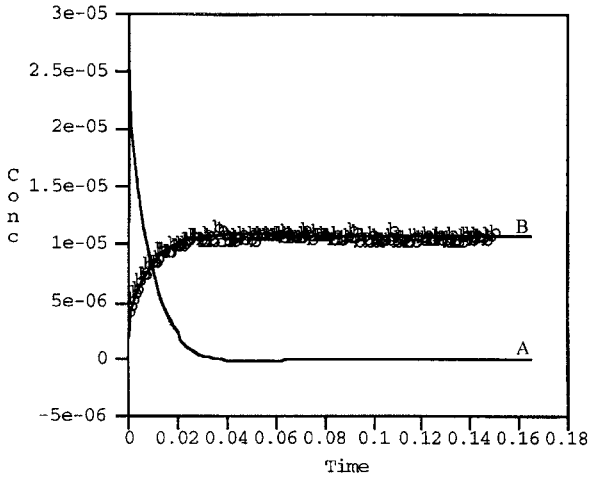

FIGURE 4: Simulation of growth of FeFz3 with apoR2 and ferrozine competing for Fe(II). Calculated (solid line marked with "B") and observed (small letter "b", from thhe observed A_{562}) $[FeFz_3]$ as a function of time. Conditions after mixing in the stopped-flow: I = 0.1 M (NaCl), 5.0 °C, pH 7.6 with HEPES 50 mM; Fz = 2.01 mM; Fe(II) = 30 μM; apoR2 29.3 μM. A value of 42 s^{-1} (obtained by observing the ferrozine reaction under the same conditions but with 29.3 μM BSA instead of apo) was assumed for k_{Fz}.

in Figure 4) were the values of k_{apo} in Table 1.

$$Fe^{II} \rightarrow Q,\ k_{Fz} \tag{17}$$

$$Q + 3Fz \rightarrow FeFz_3,\ 1 \times 10^{20} \tag{18}$$

Addition of a second iron incorporation step (eq 19) allowed us to compare its rate with that of the first step.

$$apoFe + Fe^{II} \rightarrow apoFe_2,\ k_{apoFe} \tag{19}$$

Simulation of the observed data proved impossible if k_{apoFe} was assumed to be an order of magnitude greater than k_{apo} (unless iterative refinement of k_{apoFe} reversed the order of the rate constants). Similarly, if k_{apoFe} was set equal to k_{apo}, iterative refinement of k_{apoFe} made it less than k_{apo}.

Of course, as explained in the introductory portion of this paper, agreement with the observed kinetics requires that the slow step in the formation of $apoFe_2$ be independent of $[Fe^{2+}]$ and of $[O_2]$. A conformation change (eq 20) between eq 15 and eq 19 fulfills that requirement and gives us eqs 2−4 if we set k_{conf} equal to 10 s^{-1}.

$$apoFe \rightarrow (apo')Fe,\ 10 \tag{20}$$

We therefore tested eqs 2−4 by attempting to iteratively adjust eqs 15 and 17−20 to simulate the observed time dependence of $[FeFz_3]$. However, the k_{apo} results in Table 2 do not differ appreciably from those in Table 1. Our results are thus *not inconsistent* with a k_{conf} equal to 10 s^{-1}, although they require only that uptake of the second iron be appreciably slower than that of the first. No information about k_{apoFe} is available from our experiments, as is apparent from the scatter of the "best fit" values of that parameter in Table 2.

We also attempted to model the possibility (eqs 21 and 22) that a conformational change preceded uptake of Fe^{II} by apoprotein. Very poor fits were obtained unless we allowed k_{pre} to become so large that all apoprotein was immediately converted to apo′, making the reaction effectively second order (eq 23). It seems clear that *eqs 2−4 correctly describe the uptake of Fe^{II} by apoprotein.*

$$apo \xrightarrow{k_{pre}} apo' \tag{21}$$

$$apo' + Fe^{II} \xrightarrow{k_{uptake}} (apo')Fe \tag{22}$$

$$\frac{d[Fe^{II}]}{dt} = k_{uptake}[apo''][Fe^{II}] \approx k_{uptake}[apo][Fe^{II}] \tag{23}$$

Best Value of k_{apo}. Even by iterative refinement the determination of k_{apo} requires knowledge of [apo] and $[Fe^{II}]$ at the beginning of data collection ($[apo]_0$ and $[Fe^{II}]_0$). Unfortunately the reaction proved fast enough that some $FeFz_3$ was formed and some of each reagent consumed during the dead time of the stopped-flow apparatus, so the true $[apo]_0$ and $[Fe^{II}]_0$ were not equal to $[apo]_n$ and $[Fe^{II}]_n$, the nominal concentrations of these reagents obtained by dividing the syringe concentrations by two. When $[apo]_n \approx [Fe^{II}]_n$ no good estimate of the correction was available, so estimates of k_{apo} from these runs have not been entered in Table 2 (runs 1 and 2). However, when $[apo]_n > [Fe^{II}]_n$ (runs 4−10), we assumed that the product ratio at the beginning of the reaction was approximately the same as at its end (eq 24); the resulting values of $[apo]_0$ and $[Fe^{II}]_0$ were then used

Scheme 1

Iron Uptake during Activation of R2

$$\text{apo} + \text{Fe} \longrightarrow \text{apoFe},\ k_{apo} = 10^6\ \text{M}^{-1}\text{s}^{-1}$$

$$\text{apoFe} \longrightarrow (\text{apo}')\text{Fe},\ k_{conf} = 10\ \text{s}^{-1}$$

$$(\text{apo}')\text{Fe} + \text{Fe} \longrightarrow \text{apoFe}_2,\ k_{apoFe}$$

$$\text{apoFe}_2 \xrightarrow{O_2,\ \text{fast}} \mathbf{X}\ (60\text{–}80\ \text{s}^{-1}),\ \text{then to active R2}\ (1\ \text{s}^{-1})$$

to determine k_{apo}.

$$[\text{apo}]_n - [\text{apo}]_0 = (\text{yield of apoFe})\{[\text{Fe}^{II}]_n - [\text{Fe}^{II}]_0\} = (\text{yield of apoFe})\frac{[\text{FeFz}_3]_0}{(\text{yield of FeFz}_3)} \quad (24)$$

It is also impossible to determine k_{apo} well if too little $FeFz_3$ is formed for its concentration to be measured accurately. We have therefore entered no value of k_{apo} for run 3 in Table 2, where [apo] is relatively large.

The remaining runs in Table 2 give an average value of $1.80(14) \times 10^6\ M^{-1}\ s^{-1}$.

CONCLUSIONS

Our experiments, along with the earlier work on the kinetics of formation of **X** (*12*−*15*), suggest that eqs 1−4 in Scheme 1 are correct and that k_{apo} has a value of about $1.8 \times 10^6\ M^{-1}\ s^{-1}$ at 4 °C. While it is apparent that uptake of the second iron is slower, the delay is caused by the conformational change after uptake of the first iron; the value of k_{apoFe} remains unknown.

There is other evidence that apoR2 binds a second iron more slowly than the first. Bollinger and co-workers have identified by Mössbauer spectroscopy a site (called site 2) that is preferentially occupied by the initial Fe^{2+} and becomes (a) the site of **X** with partial Fe(IV) character and eventually (b) the site Fe_B in oxidized, active R2 (*37*). Furthermore, as noted by Bollinger and co-workers, crystalline mouse R2 binds iron only to the Fe_B site (*38*), perhaps because the required conformational change is impossible in the solid state.

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SUPPORTING INFORMATION AVAILABLE

Order in [Fz] of Fe^{2+} uptake; effect of BSA on uptake of Fe^{2+} by Fz. This material is available free of charge via the Internet at http://pubs.acs.org.

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