

Mechanism and Kinetics of Iron Release from Ferritin by Dihydroflavins and Dihydroflavin Analogues[†]

Tim Jones,[‡] Rob Spencer,[§] and Christopher Walsh^{*,*#}

ABSTRACT: Dihydroflavins reductively release iron rapidly and quantitatively from purified horse spleen or horse heart ferritin. The NAD(P)H:flavin oxidoreductase from *Beneckea harveyi* is used to generate a constant concentration of dihydroflavin permitting a continuous assay for complete iron release. Sepharose-linked dihydroflavins are not competent to release ferritin iron, demonstrating that the dihydroflavin must pass through the channels of the protein shell prior to iron reduction. Several experiments fail to show any specific flavin

binding site, though dihydroflavins do display saturation kinetics with very high apparent K_m 's. The rates of iron release by a number of dihydroflavin analogues show that the electron transfer is significantly rate determining in iron release by dihydriboflavin, while diffusion of the dihydroflavin through the protein channel is slow in the release of iron by dihydroFMN. The rate of iron release is also dependent on the initial content of iron, having a maximum at 1200 iron atoms per ferritin.

Ferritin is the major eukaryotic iron storage protein. In mammals ferritin is found in all tissues but exists principally in liver, spleen, and bone marrow accounting for 15–20% of the total iron in humans (Munro & Linder, 1978). The apoprotein is a multimer of 24 similar but not identical subunits (Drysdale et al., 1977) that form a hollow sphere of approximately 450 000 daltons. This protein shell is approximately 24 Å thick with an internal diameter of 70–80 Å and is pierced by six channels. The channels are positioned along fourfold symmetry axes and are each 9–12 Å across at the outer surface and 17–20 Å across at the inner surface (Hoare et al., 1975). In the holoprotein the shell surrounds a "core" of a ferric oxyhydroxide phosphate polymer containing up to 4500 iron atoms with a molecular weight of up to 400 000 (Crichton, 1973). Ferritin serves to solubilize and sequester this iron. Good evidence exists that iron is delivered to ferritin as Fe^{II} , deposited as Fe^{III} , and released upon reduction back to Fe^{II} (Bielig & Bayer, 1955; Niederer, 1970). In this paper we are concerned with the mechanism of this reductive release of iron.

Sirivech et al. (1974) have shown that biological reductants such as thiols, ascorbate, and dihydroflavins are competent to reduce and release iron from ferritin; only dihydroflavins mediate complete iron release at a sufficient rate to be physiologically significant. We have consequently focused on the dihydroflavins, making use of a new assay system employing the *Beneckea harveyi* NAD(P)H:flavin oxidoreductase to generate and to maintain a constant concentration of dihydroflavins and dihydroflavin analogues. We have examined ferritins from different tissues and synthetic ferric oxyhydroxide cores to explore the following questions:

1. What is the overall stoichiometry of the iron reduction reaction?

2. Must dihydroflavins enter the ferritin channels to reduce and release iron? Is there an inverse relation between dihydroflavin size and the rate of iron release?

3. Is there evidence for a specific dihydroflavin binding site on the protein?

4. What is the rate-determining step in iron release? If the reductant and iron must pass through the channels, is this physical transport step slow or is the redox reaction rate limiting?

5. Are there kinetic differences in iron release from heart and spleen ferritin?

Experimental Section

Materials

Fresh horse liver and spleen, from the same animal, were obtained from Peter Deniz of Taunton, Mass. Ferritin was purified from these tissues by the modified procedure of Drysdale & Munro (1965), replacing the gel filtration on Sephadex G-200 with chromatography on Sepharose 6B, equilibrated, and run in 0.1 M Tris-HCl,¹ pH 7.4, 4 °C. Horse spleen ferritin crystallized from cadmium sulfate was obtained from Sigma Chemical Co. Heavily loaded horse spleen ferritin (3400 g-atom of Fe/mol) was kindly provided by Dr. James Drysdale.

The NAD(P)H:flavin oxidoreductase from *Beneckea harveyi* in partially purified form was the gift of Drs. James Becvar and Robert Presswood. It was further purified as in Fisher et al. (1976).

Polymers from the basic hydrolysis of ferric citrate ("synthetic cores") were prepared according to Spiro et al. (1967) and were chromatographed on Sephadex G-15 equilibrated in 10 mM sodium citrate, pH 9.0, 20 °C.

[2-¹⁴C]Riboflavin (31 Ci/mol) was obtained from Amersham/Searle, Inc. FMN was prepared from FAD (Sigma) with snake venom phosphodiesterase and chromatographed

[†] From the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. Received March 20, 1978. This work supported in part by National Institutes of Health Grants GM 21643 and 1-P07-Pr00798.

[‡] Supported in part by the Undergraduate Research Opportunities Program of M.I.T. Current address: Department of Physiology, University of California, San Francisco, California 94143.

[§] National Science Foundation predoctoral fellow, 1974–1977. Current address: Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706.

[#] Camille and Henry Dreyfus Scholar, 1976–1980.

¹ Abbreviations used: RF, riboflavin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Fl_{ox} , oxidized flavin (general); FlH_2 , dihydroflavin (general); 1-deazariboflavin, 1-carba-1-deazariboflavin; 5-deazariboflavin, 5-carba-5-deazariboflavin; 7-chlororiboflavin, 7-chloro-7,8-didemethylriboflavin; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: The Kinetics of Iron Release by Dihydroflavins.^a

flavin	$E^{\circ'}$ (mV)	from synthetic cores ($M^{-1} s^{-1}$)	from spleen ferritin			from heart ferritin		
			V_{\max} ($\times 10^2$ s^{-1})	K_m (μM)	V_{\max}/K_m ($M^{-1} s^{-1}$)	$V_{\max} \times 10^2$ (s^{-1})	K_m (μM)	V_{\max}/K_m ($M^{-1} s^{-1}$)
riboflavin	-208 ^b	160	3.0	250	120	1.3	250	52
FMN	-216 ^b	160	0.33	60	55	0.07	60	12
FAD	-219 ^b	70	0.44	150	30	0.13	150	8.7
7-chlororiboflavin	-128 ^c	56	1.1	220	50	0.50	220	23
Ia	-176 ^d	100	1.4	240	58	0.48	240	20
IIa	-196 ^d	140	2.8	250	110	0.74	250	30
3-carboxymethylumiflavin	-236 ^d	260	0.03	<i>e</i>	<i>e</i>			
3-deazariboflavin	-240 ^c	240						
1-deazariboflavin	-280 ^c	410	8.9	290	310	3.0	290	100
3-methylumiflavin			<i>f</i>	<i>f</i>	460	<i>f</i>	<i>f</i>	180

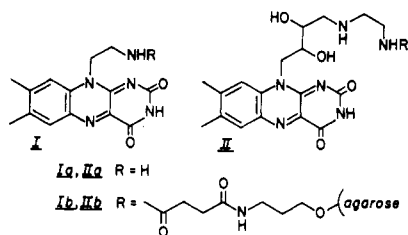
^a Rates from synthetic cores were determined under pseudo-first-order conditions at 25 μM dihydroflavin. V_{\max} is expressed as nmol of Fe^{II} released per s per nmol of initial Fe^{III} , and release from synthetic cores as this rate divided by 25 μM . ^b Loach (1968). ^c Walsh et al. (1978).

^d These were measured by redox dye equilibration as in Walsh et al. (1978). ^e No decrease in the rate of iron release was seen with dihydro-3-carboxymethylumiflavin as low as 8 μM . ^f No saturation was observed with dihydro-3-methylumiflavin as high as 200 μM .

nmol of total Fe^{III} ; 5 mM bipyridyl; 0.1 M Tris-HCl, pH 7.4). The observed release of 9.5, 20.0, and 30.5 nmol of Fe^{II} , respectively, confirms this stoichiometry.

The two reducing equivalents of dihydroflavins may be transferred to ferritin iron in either a concerted or sequential manner. To test these alternatives, ferritin was incubated with 50 μM dihydro-5-deazariboflavin; no detectable Fe^{II} was released over a 60-min period. Since dihydro-5-deazaflavins are oxidized quite readily by two electron acceptors (Fisher et al., 1976; Walsh et al., 1978) but by one electron acceptors at rates only 10^{-6} to 10^{-4} those of dihydroflavins (Walsh et al., 1978; L. Hersh, R. Spencer, & C. Walsh, unpublished observations), this result strongly suggests that dihydroflavins reduce ferritin iron in sequential one electron transfers.

Penetration of Dihydroflavins to the Ferritin Core. Reducing equivalents could pass to the ferric core of ferritin by (i) direct passage of the reductant through the six apoprotein channels to the surface of the ferric core where electron transfer takes place, (ii) transfer of electrons from the reductant to an acceptor at the outer surface of the protein shell and into the core by a protein-mediated mechanism, or (iii) transfer of electrons from the reductant to a relatively small population of ferric iron near the surface of the protein shell, which is in equilibrium with the bulk of the ferric core. To probe directly hypothesis (i), we have prepared compounds Ia, Ib, IIa, and IIb.



Dihydro-Ia and dihydro-IIa are fully competent to release Fe^{II} from both ferritin and synthetic cores (formed by the hydrolysis of ferric citrate) (Table I). Dihydro-Ib and dihydro-IIb, which are Ia and IIa in amide linkage to agarose beads release Fe^{II} from the synthetic cores but do not release any Fe^{II} from ferritin.² Since ferritin iron is reduced by a broad variety of dihydroflavins (vide infra), this incompetence of IIa and IIb suggests that the reductant must pass through the protein channels. From their 6-Å resolution electron density map, Harrison et al. (1975) propose that molecules the size of RFH_2 ,

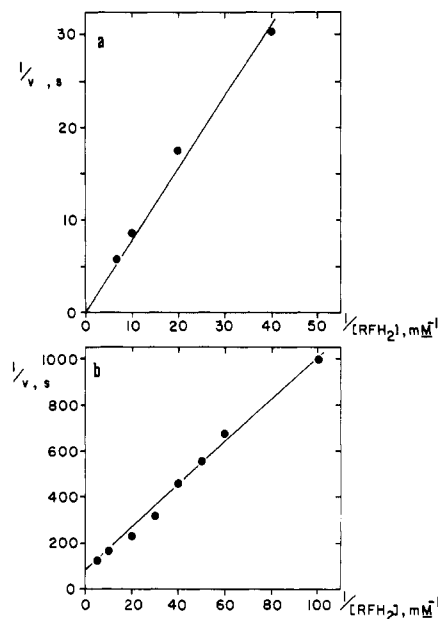


FIGURE 2: Double-reciprocal plots of the rates of iron release vs. dihydroflavin concentration. The upper figure (a) is the release from synthetic cores and the lower figure (b) is the release from horse spleen ferritin. Rates are in nmol of Fe^{II} released per s per nmol of initial Fe^{III} .

$FMNH_2$, and $FADH_2$ are able to pass through the channels, which are 9–12 Å across at their narrowest point.

Saturation Kinetics. Figures 2a and 2b are double-reciprocal plots of the rates of release of Fe^{II} from ferritin and synthetic cores at varying concentrations of dihydroflavin. As expected, release of Fe^{II} from synthetic cores shows no evidence of saturation and follows second-order kinetic dependence in dihydroflavin and iron. Release of Fe^{II} from ferritin is first order in ferritin (data not shown), but does saturate in dihydroflavin with an apparent K_m of 250 μM . The V_{\max} and K_m data for this and other dihydroflavins are collected in Table I. The values of V_{\max}/K_m are suggested as the best measure of the relative "efficiencies" of the various

² The Fe^{II} is released from synthetic cores by the flavin-agarose at approximately 20% the rate with dihydroflavin at the same total flavin concentration. With ferritin in the discontinuous assays, no Fe^{II} is seen after 75 min of incubation.

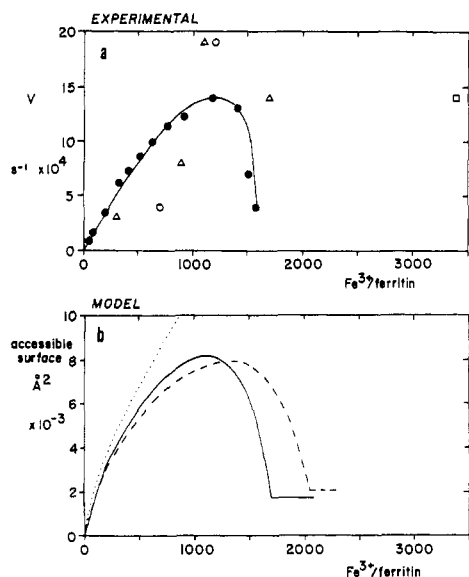


FIGURE 3: The relation between ferritin iron load and the rate of iron release by dihydroflavin. [Upper figure (a)] Open symbols are the initial rates of Fe^{II} release from ferritin samples of varying iron loading: (Δ) horse spleen ferritin; (\circ) horse heart ferritin; and (\square) heavily loaded horse spleen ferritin provided by Dr. J. Drysdale. In all cases the dihydroflavin concentration was $50 \mu\text{M}$. Solid circles (\bullet) are rates obtained by differentiation of the continuous assay of Figure 1. [Lower figure (b)] The accessible surface area of the ferritin iron core (A) was calculated as in Appendix I, as a function of iron content (N) for $(--)$ $R \rightarrow \infty$, i.e., no protein shell; $(- - -)$ for $R = 43 \text{ \AA}$ and $a = 6(18.5)^2 \text{ \AA}^2$ (Hoare et al., 1975), and $d = 8 \text{ \AA}$. The closest fit to the experimental data ($—$) was obtained with $R = 39 \text{ \AA}$, $a = 6(18.5)^2 \text{ \AA}^2$, and $d = 5 \text{ \AA}$.

dihydroflavins.³ The saturation kinetics observed can be a result of either (i) one or more specific binding sites for dihydroflavins on the protein shell, or (ii) a nonspecific, partially rate-determining step such as diffusion of dihydroflavin through the protein channels. We present evidence below inconsistent with the first possibility.

Equilibrium Dialysis. A series of equilibrium dialysis experiments with ferritin and apoferritin vs. oxidized and reduced $[2\text{-}^{14}\text{C}]$ riboflavin and reduced $[2\text{-}^{14}\text{C}]$ FMN failed to show any affinity of the proteins for the flavins. The concentrations of protein and specific activity of the flavins were such that any dissociation constant $\leq 1 \text{ mM}$ could easily have been measured. This supports the hypothesis that the observed kinetic saturation and apparent K_m 's of dihydroflavins do not reflect dissociation constants (K_d 's) and that there is no specific flavin or dihydroflavin binding site on ferritin.

Kinetic Competition by Dihydro-5-deazaflavins. Since dihydro-5-deazaflavins are not able to reduce ferritin iron (vide supra), they might be expected to compete nonproductively with dihydroflavins for any such binding site on ferritin. With dihydroflavin at $10 \mu\text{M}$, dihydro-5-deazariboflavin at $65 \mu\text{M}$ did not affect the rate of iron release from ferritin. Similarly, the rate of ferritin iron release by $10 \mu\text{M}$ dihydroFMN was not affected by up to $120 \mu\text{M}$ dihydro-5-deazaFMN. This demonstrates that no dihydroflavin sites kinetically relevant to ferritin iron reduction exist that bind dihydro-5-deazariboflavin or dihydro-5-deazaFMN with K_d 's less than approximately 1 mM and 0.5 mM , respectively.

The Dependence of Ferritin Iron Reduction Rates on Extent of Iron Loading. A sigmoidal pattern of iron release is always

seen in assays of complete iron release from ferritin (e.g., Figure 1). In the complete reduction of synthetic ferric cores, no initial slow phase is seen, and the reaction is simply pseudo-first-order in remaining ferric iron.⁴ The origins of the slow phase in the ferritin kinetics are complex, though several possibilities can be eliminated in turn. First, our strict anaerobiosis and the absence of the slow phase with synthetic cores preclude the possibility that the slow phase is due to dihydroflavin consumption by residual oxygen (Crichton et al., 1975, 1977). Second, the slow phase is not the result of a slow approach to a kinetic steady state. Sirivech et al. (1974) showed that a steady state is achieved under comparable conditions within 2 s. Third, the slow phase is probably not representative of "loosely bound iron" or other obvious iron heterogeneity (Dognin & Crichton, 1975), since this phase releases a constant 12–15% of the total ferritin iron, independent of sample origin or preparation. We suggest that this slow phase of ferritin iron release may be a function of both (i) an inherent lesser chemical reactivity of Fe^{III} at the outer surface of the ferritin iron cores, vs. that buried inside,⁵ and (ii) hindered access of the reductant (once inside the ferritin) to the ferric core by the protein shell, that is particularly apparent during initial iron release. The latter hypothesis is elaborated below in the context of earlier studies (Hoy et al., 1974) and an extended mathematical model.

Several experimenters (Harrison et al., 1974, 1975; Hoy et al., 1974) have suggested that both the rates of deposition of iron into ferritin ($\text{Fe}^{\text{II}} \rightarrow \text{Fe}^{\text{III}}$, with molecular oxygen as oxidant) and of removal of iron from ferritin are directly proportional to the available surface area of the ferric core within the apoprotein shell. These studies of iron release involved either nonreductive removal of iron by chelating agents or reduction with such reducing agents as thiols. These methods are both too slow to be of physiological importance (half-times of hours to days) and cannot remove 100% of ferritin iron. We have accordingly examined this point with the anaerobic dihydroflavin assay system.

Experiments were done in two ways: first, ferritin samples were prepared with differing iron contents, and initial rates of iron release were measured in the standard assay. Second, assays of complete iron release (e.g., Figure 1) were used to compute the rate of iron release as a function of remaining ferric iron throughout the assay. Figure 3a shows the results of both techniques. The rate of iron release increases with increasing iron content to a maximum near 1200 g-atom iron per mol of ferritin and then falls to a plateau rate for loadings greater than about 2000 g-atom per mole, in agreement with the previous, slower assays of Hoy et al. (1974), Harrison et al. (1974), and Miller & Perkins (1969).

Appendix I details the calculation of the available surface area of a spherical ferric core within a spherical protein shell pierced by six channels. This model extends that of Hoy et al. (1974). The model contains only one unknown parameter, the "exclusion distance" d , which is the minimum gap through which a reductant molecule may pass. With values of d between 5 and 8 \AA and the other parameters taken from the crystal structure of apoferritin (Hoare et al., 1975; Harrison et al., 1975), a close correlation is obtained between the calculated available core surface area and the experimental rates

⁴ This is expected since the reaction is first order in both FIH_2 and ferritin and the generating system holds FIH_2 constant.

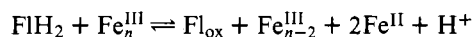
⁵ This could be due to the predominance of phosphate or other anions at the surface of the ferritin cores (Spiro et al., 1967) or to differences in hydration ("aging") between the core surface and interior (Sommer et al., 1973).

³ The concentrations of dihydroflavins in vivo are likely to be far below the K_m values in Table I, so that ferritin iron reduction by dihydroflavins will take place under second-order conditions.

of iron release. We therefore propose that in the release of ferritin iron by dihydroflavins the rate is proportional to the available surface area of the ferritin core.

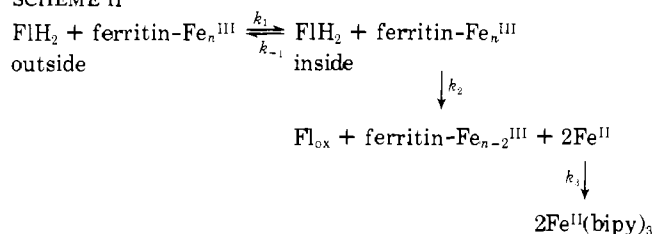
Rate Determining Steps in Ferritin Iron Release. To determine the rate-limiting steps in the reductive release of ferritin iron we have used a number of flavin analogues which may be conveniently divided into two categories. The first series consists of flavins that share a common redox potential but that differ in size and/or charge, and the second series consists of flavins that are essentially isosteric but that differ markedly in redox potential.

Synthetic Cores. It was essential to establish rate vs. redox potential relations in the absence of any influence of the apoferritin shell. The "synthetic cores" of ferric oxyhydroxide polymer were prepared primarily for this reason. As is evident in Table I, all of the dihydroflavins with redox potentials near -208 mV (lumiflavin, riboflavin, FMN, FAD) reduce the synthetic cores at comparable rates. The charge and size of dihydroFMN and dihydroFAD are apparently no hindrance in their reduction of ferric iron at the cores' surface. On the other hand, as Table I and Figure 4 show, the dihydroflavins with altered redox potentials reduced the synthetic cores at different rates. These data follow a linear free energy-rate relation, with the strongest reductants reducing the core iron at the fastest rates. Similar relationships for flavin analogues have been seen in their reactions (enzymatic and nonenzymatic) with nitroxides, molecular oxygen, dihydronicotinamides, and dihydro-5-deazaflavins (Chan & Bruice, 1977; Blankenhorn, 1976; Walsh et al., 1978). This correlation implies that the redox reaction between dihydroflavin and ferric iron is substantially rate limiting in the reduction of synthetic cores.⁶ The minimal kinetic scheme for the synthetic core reaction is then:



Ferritin. The kinetics of iron release from ferritin itself are more complex. A minimal kinetic scheme for the overall reaction can be simplified to Scheme II (see Appendix II),

SCHEME II



where "inside" and "outside" refer to the inside or outside of the apoferritin shell.

Polyanionic dihydroflavins (FMNH_2 , FADH_2 , 3-carboxymethylumiflavin H_2) release iron from ferritin at much slower rates than other dihydroflavins (Table I and Figure 4). Since dihydroflavin anionic substituents do not significantly slow the rates of synthetic core reduction (Table I), we suggest that k_1 , and/or k_{-1} , of Scheme II are specifically lower for these dihydroflavins. Such an electrostatic hindrance to diffusion is supported by the acidic pI of ferritin (i.e., that there may be aspartate or glutamate residues in or near the channels) and the findings of May & Fish (1977) that the diffusion of acetate

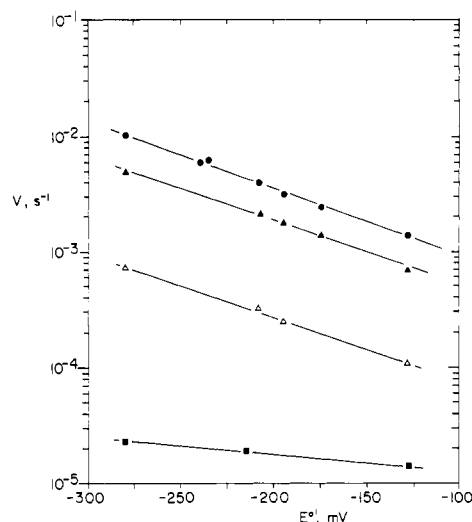


FIGURE 4: The rates of iron release from synthetic cores and ferritin vs. flavin analogue redox potentials. The iron release from synthetic core (●) was accomplished with (from left to right) 1-deazariboflavin, 3-deazariboflavin, 3-carboxymethylumiflavin, riboflavin, Ia, Ia, and 7-chlororiboflavin. The iron release from horse spleen ferritin (▲) was measured with the same flavins, excepting 3-deazariboflavin and 3-carboxymethylumiflavin. The release from horse heart ferritin (△) was with the same analogues, excepting Ia. The iron release from horse spleen ferritin with FMN and FMN analogues (■) was with (from left to right) 1-deazaFMN, FMN, and 7-chloroFMN. All dihydroflavins were at $25 \mu\text{M}$, and all rates are expressed as nmol of Fe^{II} released per s per nmol of initial Fe^{III} .

into apoferritin is much slower than that of glucose, methanol, or methylammonium ion.

Figure 4 shows that the release of iron from ferritin by those dihydroflavins with neutral or cationic substituents (points ▲) follows a rate vs. redox potential correlation with the same slope ($d \log k / dE^\circ$) as that observed for the synthetic cores. The absolute rates, expressed as second-order rate constants in total ferric iron and dihydroflavin, are approximately two-fold lower for ferritin than the synthetic cores. The identity of the $d \log k / dE^\circ$ slopes leads us to propose that the redox step (k_2 , Scheme II) is significantly rate determining for dihydroflavin-mediated iron release from ferritin, as it is for release from synthetic cores. We suggest that the absolute rate differences reflect different effective surface areas of the synthetic vs. ferritin cores. The "masking" of the ferritin core in our model (Appendix I) accounts for a factor of about two in rate.

The rates for ferritin iron release by dihydroFMN and its analogues (Figure 4, ■) strongly suggest that some step other than k_2 has become partially rate determining for these polyanionic dihydroflavins. Both the absolute rates of iron release and the $d \log k / dE^\circ$ slope are less than those of neutral or cationic dihydroflavin-mediated release. This suppression of E° dependence suggests a change in rate-determining step with dihydroFMN. We conclude that it is the redox chemistry (k_2 , Scheme II) that is largely rate determining in the release of ferritin iron by dihydroflavin, and a physical step, possibly dihydroflavin diffusion through the protein channels (k_1 and k_{-1}) that is largely rate determining in the release by dihydroFMN and dihydroFAD.

Ferritins from Different Tissues. Table I presents data from ferritins isolated from the heart and spleen of the same animal. The apparent K_m 's for all dihydroflavins are essentially identical for both ferritins, and the V_{max}/K_m values for heart ferritin are consistently ca. 0.3 those for spleen ferritin. When V_{max}/K_m values are calculated on the basis of protein con-

⁶ The slope of the correlation is $170 \text{ mV}/\log k$, so that for the overall two electron reaction, $\Delta G^\ddagger = 0.17 \Delta G^\circ$. This significant dependence of ΔG^\ddagger on ΔG° can exist only when the step involving the measured ΔG° (the redox step) is significantly rate determining.

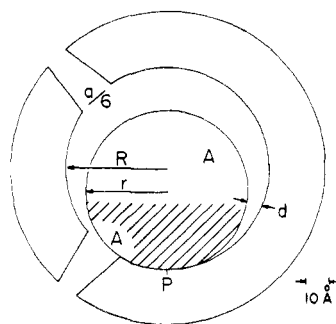


FIGURE 5.

centration, rather than total iron concentration, heart ferritin rates become approximately 0.5 those of spleen ferritin. This change is due to the different iron contents of the ferritins as isolated: heart ferritin contained 900 g-atom of iron per mol of apoferritin, and spleen ferritin 1200 g-atom of iron per mol. These V_{\max}/K_m data may be consistent with the findings of Drysdale et al. (1977) that heart apoferritin contains a larger fraction of "heavy" subunits (21 000 daltons) than spleen apoferritin, which is predominantly composed of "light" subunits (19 000 daltons). The larger subunits of heart ferritin may restrict dihydroflavin diffusion to the ferric core more than those of spleen ferritin, and thereby alter V_{\max}/K_m for all dihydroflavins.

Another difference between heart and spleen ferritins is the extent of iron loading at the maximum rate of iron release. For spleen ferritin the maximum rate is observed when the ferric core contains approximately 1200 g-atom of Fe per mol; the value for heart ferritin is consistently 900 g-atom Fe per mol. We cannot explain this difference without overextending our simple models.

The maximum rate of iron release from spleen and heart ferritin by dihydroriboflavin may be expressed as the number of Fe^{II} that must pass through each protein channel per second.

$$\left(0.0037 \frac{\text{nmol of Fe}^{\text{II}}}{\text{nmol of Fe}^{\text{III}}_{\text{initial}} \text{ s}}\right) \times \left(1600 \frac{\text{nmol of Fe}^{\text{III}}_{\text{initial}}}{\text{nmol of ferritin}}\right) \left(\frac{1}{6} \frac{\text{nmol of ferritin}}{\text{nmol of channels}}\right)$$

For spleen ferritin this is to give 1.0 Fe^{II} leaving per second per channel. For heart ferritin the calculation gives 0.24 Fe^{II} per second per channel. With the stoichiometry of 2 Fe^{III} reduced per dihydroriboflavin, the rates of dihydroriboflavin passage through the channels must be at least half these values.

Physiological Significance

Given that dihydroflavins can completely and rapidly release iron from isolated samples of ferritin, one can inquire whether this process may be of physiological significance in mobilization of ferritin-bound iron for hemoglobin and myoglobin synthesis. Iron atoms associated with ferritin are sequestered physically, and as Fe^{III} are highly insoluble in aqueous solutions at physiological pH ($(\text{FeOH})_3$; $K_{\text{sp}} = 10^{-39} \text{ M}^3$; Spiro, 1977). Dihydroflavins can penetrate the channels of the apo-protein shell and solubilize ferritin iron as Fe^{II} by electron transfer ($(\text{FeOH})_2$; $K_{\text{sp}} = 10^{-15} \text{ M}^2$; Spiro, 1977). Are dihydroflavins likely to be freely diffusible components with finite lifetimes in aerobic mammalian cells? This is unresolved. Concentrations of flavins in horse tissues (the source of ferritins used in this paper) are not available, but total concentrations for rat tissue are as follows: (1) liver—28–75 μM FAD, 15–18 μM FMN, 2.1 μM RF; (2) heart—18–45 μM FAD, 3.3–4.4

μM FMN, 0.3 μM RF; (3) spleen—13 μM FAD, 0.4 μM FMN, 2.9 μM RF (Yagi, 1977; McCormick, 1975). Most of the tissue FMN and FAD (and possibly riboflavin) are tightly bound to specific proteins and are not freely diffusible. In addition most flavin-linked redox enzymes exist as holoenzymes. The flavins do not dissociate at the end of each catalytic cycle. The *B. harveyi* oxidoreductase is unusual since it does generate dihydroflavins free in solution. The mammalian counterparts may exist. Sirivech et al. (1977) have reported partial purification of a NADH:FMN oxidoreductase from rat liver. This rat liver activity is reputedly lower in riboflavin-deficient or iron deficient rats (Sirivech et al., 1977; Zaman & Verwilghen, 1977). Yubisui et al. (1977) have purified a methemoglobin reductase from human erythrocytes which is, in fact, an NADPH:flavin oxidoreductase. Similar reduction of Fe^{III} to Fe^{II} in ferritin could occur.

In pathological conditions such as Cooley's anemia and thalassemia, chronic transfusions generate secondary hemochromatosis with heavily overloaded ferritin in tissue and serum. Iron chelating drugs have great thermodynamic affinity for iron but may be limited by kinetic inaccessibility of ferritin iron (Anderson & Hiller, 1975; Sirivech et al., 1974). Riboflavin and riboflavin analogues with strongly negative redox potentials may warrant investigation for possible acceleration of iron release.

Acknowledgments

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Appendix I: The Available Surface Area of a Sequestered Iron "Core"

This calculation is based on the idealized ferritin model shown. In Figure 5, a spherical iron core of radius r containing N iron atoms is sequestered within the ferritin protein shell of interior radius R , being held to the surface at point P . For evidence that ferritin iron is "fastened" in this sense see Stuhmann et al. (1975). The protein shell is pierced by six channels, two of which are shown, with total cross-sectional area at the interior surface a . The available surface area A is taken to be that portion of the total iron core surface that is accessible to a molecule of linear dimension greater than or equal to d ; thus all of the iron core closer to the protein shell than d is not available to react with such a molecule. This inaccessible portion of the core is shaded in the figure. Note that at the channels the core is always accessible. The solution for A is given by

$$A = 2\pi r^2(1 + \xi) + \frac{ar^2}{2R^2}(1 - \xi)$$

where

$$\xi = \frac{r(R - r - d) - d^2/2}{r(R - r - d) + dR}$$

$$r = \left(\frac{3}{4\pi\rho} N\right)^{1/3}$$

