Fung, L. W.-M. (1981b) *Biophys. J. 33*, 253.
Fung, L. W.-M., & Simpson, M. J. (1979) *FEBS Lett. 108*, 269.
Fung, L. W.-M., & Ostrowski, M. S. (1982) *Am. J. Hum. Genet. 34*, 469.
Fung, L. W.-M., Lin, K. L. C., & Ho, C. (1975) *Biochemistry 14*, 3424.
Gopinath, R. M., & Vincenzi, F. F. (1979) *Am. J. Hematol. 7*, 303.
Hosey, M. M., & Tao, M. (1976) *Nature (London) 263*, 424.
Lessin, L. S., Kuranstan-Mills, J., Wallas, C., & Weems, H. (1978) *J. Supramol. Struct. 9*, 537.
Lubin, B., Chiu, D., Bastacky, J., Roelofsen, B., & Van-Deenen, L. L. M. (1981) *J. Clin. Invest. 67*, 1643.
Lux, S. E. (1979) *Semin. Hematol. 16*, 21.
Lux, S. E., John, K. M., & Karnovsky, M. J. (1976) *J. Clin. Invest. 58*, 955.
Minton, A. P. (1977) *J. Mol. Biol. 89*, 110.
Noguchi, C. T., & Schechter, A. N. (1981) *Blood 58*, 1057.
Perutz, M. F., Liquori, A. M., & Eirich, F. (1951) *Nature (London) 167*, 929.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346.
Rosenberry, T. L., Chen, J. F., Lee, M. M. L., Moulton, T. A., & Onigman, P. (1981) *J. Biochem. Biophys. Methods 4*, 39.
Salhany, J. M., & Shaklai, N. (1979) *Biochemistry 18*, 893.
Schroeder, W. A., & Huisman, T. H. J. (1980) *The Chromatography of Hemoglobin*, Marcel Dekker, New York.
Shaklai, N., Sharma, V. S., & Ranney, H. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 65.
Smith, C. M., Kuettner, J. F., Tukey, D. P., Burris, S. M., & White, J. G. (1981) *Blood 58*, 71.
Steck, T. L., & Kant, J. A. (1974) *Methods Enzymol. 32B*, 172–180.
Stryer, L. (1981) *Biochemistry*, W. H. Freeman, San Francisco.
VanAssendelft, O. W., & Zijlstra, W. G. (1975) *Anal. Biochem. 69*, 43.

Mercuric Reductase from R-Plasmid NR1: Characterization and Mechanistic Study†

Stephen J. Rinderle, James E. Booth, and Jeffrey W. Williams*

ABSTRACT: Mercuric reductase, a flavoprotein which catalyzes the NADPH-dependent reduction of mercuric ion to metallic mercury, has been purified from *Escherichia coli* containing the cloned mercury resistance genes from plasmid NR1. The purification, which involves Blue Dextran affinity chromatography and ion exchange, gives a 1000-fold purification with an overall yield of 30%. The purified enzyme has a molecular weight of 110 000 and is composed of two identical subunits of 56 000 each. The enzyme was found to be incapable of reducing any of the other metal ions tested although Cd, Ag, Cu, and Au were shown to be potent inhibitors. Anaerobic titration of the enzyme with NADPH indicated that the enzyme-bound FAD could not be fully reduced to $FADH_2$ unless arsenite was included in the reaction mixture. In the absence of arsenite, NADPH formed a charge-transfer complex with partially reduced enzyme with an absorbance maximum around 540 nm. The similarity of these spectra with glutathione reductase suggested the presence of oxidation–reduction active cysteine residues at the active site. This was verified by the appearance of two additional thiols in NADPH, reduced enzyme. In the presence of Hg^{2+}, the purified enzyme requires ethylenediaminetetraacetic acid (EDTA) or thiol reagents for activity. With EDTA, there is an initial, rapid reaction velocity which slowly ($t_{1/2}$ = 0.14 s) decreases to no detectable rate, indicating the slow formation of an irreversibly inhibited complex. The completely inhibited enzyme can be slowly reactivated by the addition of 2-mercaptoethanol. In the presence of thiol reagents there was marked biphasic kinetics characterized by an initial rapid reaction velocity which slowly reaches a much slower steady-state rate. This was shown to be a hysteretic phenomenon induced by sulfhydryl compounds. These results are discussed in terms of the proposed mechanism.

The enzyme mercuric reductase (reduced NADP:mercuric ion oxidoreductase), which catalyzes the NADPH-dependent reduction of mercuric ion to elemental mercury, occupies a key position in the mercury detoxification system found in many bacteria (Summers & Silver, 1978). Organisms which possess the required plasmid-encoded genes for mercuric reductase are able to reduce mercuric ion to elemental mercury which is volatile. The volatile mercury evaporates from the growth medium. Studies aimed at understanding the properties of mercuric reductase will give insight into the molecular mechanism of this rather unique biological detoxification system. This information will be useful from the standpoint of mercury toxicology and the overall mercury cycle in our environment. Mercuric reductase has previously been isolated from *Pseudomonas* K62 (Furukawa & and Tonomura, 1972; Tezuka & Tonomura, 1976, from two plasmid-bearing *Escherichia coli* (Izaki et al., 1974; Schottel, 1978), and most recently from a strain of *Pseudomonas aeruginosa* carrying the plasmid pVS1 (Fox & Walsh, 1982). From these studies the enzyme has been found to contain FAD, utilize NADPH as an electron-donating cofactor, and require thiol reagents for activity. Fox & Walsh (1982) have also shown that mercuric reductase appears to have a mechanism similar to lipoamide dehydrogenase and glutathione reductase. These flavoproteins catalyze the transfer of electrons between pyridine nucleotides and disulfide substrates through the oxidation–

† From the College of Pharmacy, The Ohio State University, Columbus, Ohio 43210. *Received July 30, 1982.* This work was supported in part by Grant ES02701 from the National Institutes of Health.

0006-2960/83/0422-0869$01.50/0

reduction of an active site cystine residue.

This report describes the purification of mercuric reductase from *E. coli* containing the plasmid pRR130. This plasmid has been studied extensively (Jackson & Summers, 1982; Nakahara et al., 1979) and is thus genetically well characterized. In addition to the purification, this paper describes detailed studies on the substrate specificity and molecular mechanism of this enzyme. In particular, the role of thiol reagents is examined and discussed in terms of the proposed mechanism. The results presented indicate that mercuric reductase demonstrates hysteretic behavior which is dependent upon the presence of thiols.

Experimental Procedures

Materials

E. coli KP245 harboring the cloned plasmid pRR130 which contains the *mer* resistance genes of the R-plasmid NR1 was a gift from Dr. R. Rownd. DEAE-agarose was from Bio-Rad Laboratories, Sephadex resins were from Pharmacia Fine Chemicals, and Blue Dextran–Sepharose was prepared according to the procedure of Ryan & Vestling (1974). NADase was from Sigma. All other chemicals were of the highest grade available from commercial sources.

Methods

Enzyme Assays. The activity of mercuric reductase was routinely determined spectrophotometrically at 340 nm and 30 °C in 50 mM sodium phosphate, pH 7.0, 100 μM $HgCl_2$, 100 μM NADPH, 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM 2-mercaptoethanol. EDTA was found to interfere with the kinetic studies involving the various metal ions and was therefore eliminated from the reaction mixture during these experiments. Progress curves were normally obtained by continuously monitoring the disappearance of NADPH after initiation of the reaction by the addition of mercuric reductase. Reaction mixtures contained buffer, 0.5 mM EDTA, 100 μM NADPH, and various concentrations of $HgCl_2$ or 2-mercaptoethanol.

Enzyme Preparation. The bacteria were grown in Difco lactose–broth (13 g/L), sodium phosphate, monobasic (3.4 g/L), sodium phosphate, dibasic (5.7 g/L), thymine (4 mg/L), and $HgCl_2$ (27 mg/L) at 37 °C in a New Brunswick Scientific fermentor (Model FS-314) in 30-L batches. Normally, with a 2% inoculum, cells were harvested after 8 h with a Sharples T-1 continuous flow centrifuge, washed with buffer, and stored at −20 °C. The frozen cells were suspended in 5 volumes of 50 mM phosphate buffer (pH 7.0) containing 0.5 mM EDTA and 0.2 mM $MgSO_4$ and disrupted in a French press at 20000 psi. All further operations were performed at 4 °C. The cell debris was removed by centrifugation at 48000*g* for 20 min. The extract was made 1.5% (w/v) in streptomycin sulfate, and the precipitate was removed by centrifugation at 48000*g* for 15 min. The supernatant solution was applied directly to a Blue Dextran–Sepharose column and washed with buffer until protein no longer eluted. The enzyme was eluted with 1.0 M NaCl and concentrated on an Amicon PM-10 membrane. The enzyme was then passed through a Sephadex G-100 column (2.5 × 100 cm), and the active fractions were pooled and applied to another Blue Dextran–Sepharose column. After the column was washed with several column volumes of buffer, enzyme was eluted with 0.2 mM NADPH. After concentration, the enzyme solution was fractionated on a column (0.9 × 30 cm) of DEAE-agarose. During this procedure the enzyme was separated into two fractions. The first fraction eluted from the column after extensive washing with 50 mM phosphate buffer, pH 7.0. The second fraction eluted in a linear salt gradient (0–0.2 M NaCl, 70 mL). The most active of both fractions were pooled and concentrated.

Protein Concentration. Protein concentration was determined by the procedure of Lowry et al. (1951) with bovine serum albumin (BSA) as a standard. The concentration of mercuric reductase was determined from the flavin content, using an extinction coefficient of 11.3 mM^{-1} cm^{-1} at 455 nm which was verified by identical absorbance measurements before (455 nm) and after (448 nm) boiling for 15 min.

Molecular Weight Determination. The molecular weight of the native enzyme was determined by gel filtration through a Sephadex G-200 column (0.9 × 30 cm) equilibrated in the phosphate buffer above. The molecular weight standards were the following: β-galactosidase, M_r 520000; alcohol dehydrogenase, M_r 148000; alkaline phosphatase, M_r 80000; BSA, M_r 68000; pepsin, M_r 35000. The subunit molecular weight was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis by using the procedure of Weber & Osborn (1969) and Bio-Rad low molecular weight standards.

Anaerobic Titrations. The anaerobic titration of mercuric reductase with NADPH was performed in rubber-stoppered cuvettes. All solutions were flushed for 15 min with nitrogen which was passed through a vanadous sulfate amalgamated zinc solution to remove oxygen (Meites & Meites, 1948). Additions were done anaerobically by adding a concentrated solution of the reagent from a glass syringe.

Data Analysis. Enzyme inhibition which is slowly expressed such as that seen with mercuric reductase in the presence of Hg–EDTA has been discussed previously by Cha (1976) and Williams & Morrison (1979). However, unlike those previous discussions, Hg–EDTA is also a substrate, and the inhibition is irreversible. In this case, the rate of the enzyme catalyzed reaction (v) will vary with time (t) according to

$$v = v_0 \exp(-kt) \tag{1}$$

In eq 1, v_0 is the initial observed velocity extrapolated to zero time. Integration of eq 1 with respect to time gives eq 2, which

$$P = \frac{v_0}{k}[1 - \exp(-kt)] \tag{2}$$

is an expression of product concentration (P) as a function of time. The exact expression defining k, an apparent first-order rate constant, and v_0 will vary with the mechanism of the inhibition. Equations 3 and 4 may be derived to describe the

$$v_0 = \frac{VA}{K_a + A + A^2/K_i} \tag{3}$$

$$k = k_7 \frac{A/K_i}{1 + A/K_a + A/K_i} \tag{4}$$

case where a substrate may also act as a competitive inhibitor, as expected in a ping-pong mechanism (Cleland, 1979), and the inhibited form of the enzyme subsequently interacts slowly to form an irreversible, enzyme–inhibitor complex (see Scheme I). In eq 3 and 4, V is V_{max}, A is the concentration of substrate and K_a its Michaelis constant, K_i is the dissociation constant of the rapidly formed, inhibitory enzyme–substrate complex, and k_7 is the first-order rate constant for the irreversible inhibition of the enzyme.

As shown by Frieden (1979), the velocity of any enzyme reaction that exhibits hysteretic behavior can be described by the general equation

$$v = v_s + (v_0 - v_s) \exp(-k't) \tag{5}$$

where v, v_0, and t are defined as above, k' is the apparent first-order rate constant for the transition, and v_s is the final

Table I: Purification of Mercuric Reductase[a]

fraction	volume (mL)	act. (units)	protein (mg)	sp act.	fold purification
crude extract	83	97	5163	0.0189	
streptomycin sulfate	63.5	81	4318	0.0188	
Blue Dextran–Sepharose I	158	53	512	0.104	5.5
Sephadex G-100	55	34	140	0.242	12.8
Blue Dextran–Sepharose II	49	33	6.0	5.5	291
DEAE-agarose (washthrough)	53	28	1.4	20.5	1085

[a] Represents the purification starting from a 30-L batch of cells.

steady-state (linear) velocity following the burst or lag. Similar to eq 2 above, integration of eq 5 yields eq 6, which can be

$$P = v_s t - \frac{1}{k'}(v_s - v_0)[1 - \exp(-k't)] \quad (6)$$

used for progress curve analysis. If the hysteretic behavior is due to two different forms of the enzyme, separate kinetic constants for each form can be determined from eq 6 by substituting the appropriate Michaelis–Menten expression for v_0 and v_s.

Progress curve data were obtained by collecting 12–36 points from each curve on a Cary 219 spectrophotometer interfaced to a Hewlett-Packard 2115A minicomputer. These data were then directly transferred to an IBM 4331 and fitted to eq 1–4 by using the STEPIT computer program described previously by Williams et al. (1980). Kinetic constants were obtained from reaction velocity data by computer fitting to the appropriate rate equations by using the programs of Cleland (1967).

Results

Enzyme Purification. The results of a purification (30 L of cells) are summarized in Table I. Mercuric reductase in this organism appears to make up about 0.1% of the soluble protein, thus requiring a substantial purification (>1000-fold). Despite the large purification required, the procedures developed result in a reasonable overall yield (28%). Although Blue Dextran–Sepharose is believed to behave as an affinity resin for pyridine nucleotide utilizing enzymes, we found the enzyme required a further fractionation on DEAE-agarose to achieve homogeneity. It was noted that the enzyme eluted from the ion-exchange column in two fractions, the first fraction coming through the column in the buffer wash and the second eluting with the salt gradient. The relative amount of enzyme found in each fraction was not constant and was different in each enzyme preparation. The two fractions had marked differences in their mobility on polyacrylamide gels run under nondenaturing conditions. However, when the two fractions were run on sodium dodecyl sulfate (NaDodSO$_4$)–polyacrylamide gels only a single band of similar molecular weight was observed. These results are consistent with the observation by Fox & Walsh (1982) that NADP appears to be associated with some fraction of the enzyme molecules. The binding of NADP would make the protein more negative in charge and would explain our results. The enzyme fraction which did not bind to the DEAE column was found to yield a spectrum similar to that seen for NADP-free enzyme (Fox & Walsh, 1982) and was thus used in all spectrophotometric studies.

The molecular weight of the native enzyme as determined from gel filtration was 110 000 ± 10 000 while a single band of protein was detected with a molecular weight of 56 000 on sodium dodecyl sulfate–polyacrylamide gels (Figure 1). These results suggest that mercuric reductase from *E. coli* KP245 has a dimeric structure, similar to that described for the enzyme from plasmid pVS1 (Fox & Walsh, 1982), but in contrast to the trimer proposed for the enzyme from plasmid R831 (Schottel, 1978). This difference may be due to the existence of two immunologically distinct types of mercuric reductase from Gram-negative sources, where one type seems to be dimeric and the other trimeric in structure (T. Kinscherf and S. Silver, unpublished result). The heavier (M_r 62 000) of the two protein bands previously observed on NaDodSO$_4$ gels with mercuric reductase from other sources was missing in our preparation. Apparently, the relative amounts of the two enzyme forms is dependent on the age of the sample and the storage procedure, although the exact relationship between the two forms is unknown. In any case, both forms appear to be equally active (Fox & Walsh, 1982).

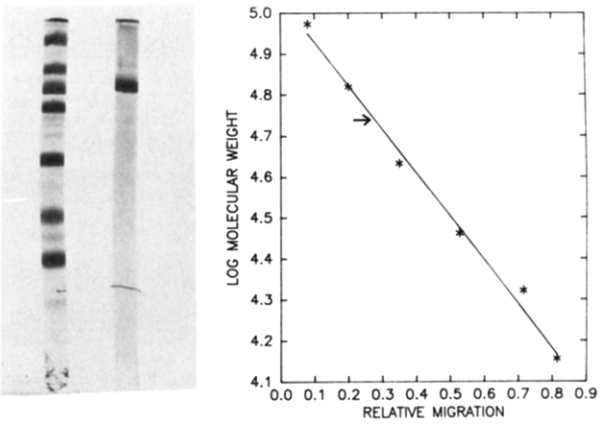


FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gel on the left contains mercuric reductase and the Bio-Rad low molecular weight standards. The other gel contains 25 μg of purified mercuric reductase. Both samples were electrophoresed as described under Methods on 10% gels. The dye front is marked by wire. On the right is a plot of these results. The arrow indicates the position of the protein band of mercuric reductase.

Table II: Metal Ion Specificity

compound	inhibitor	type of inhibition[a]	K_{is} (μM)[b]	K_{ii} (μM)[b]
$NiSO_4$	no			
$Co(NO_3)_2$	no			
$MnSO_4$	no			
$FeSO_4$	no			
$ZnSO_4$	no			
$Cr_2O_7^{2-}$	no			
$VOSO_4$	no			
$Pb(C_2H_3O_2)_2$	no			
$Fe(CN)_6^{3-}$	no			
$CdCl_2$	yes	comp	17.6 ± 2.1	
$AgNO_3$	yes	comp	1.2 ± 0.4	
$CuCl_2$	yes	comp	76 ± 7	
$CuSO_4$	yes	comp	14.2 ± 1.6	
$AuCl_3$	yes	noncomp	46 ± 26	32 ± 8

[a] Observed inhibition with Hg(II) as the varied substrate: comp, competitive; noncomp, noncompetitive. [b] K_{is} and K_{ii} are dissociation constants determined from the slope and intercept terms, respectively.

Metal Ion Specificity. A variety of metal ions were examined for their effect on mercuric reductase (Table II). None of the compounds tested were found to be substrates for the enzyme. However, a number of the metal ions were shown to be effective inhibitors. When Hg(II) was the varied substrate, all of the inhibitors were of the competitive type except

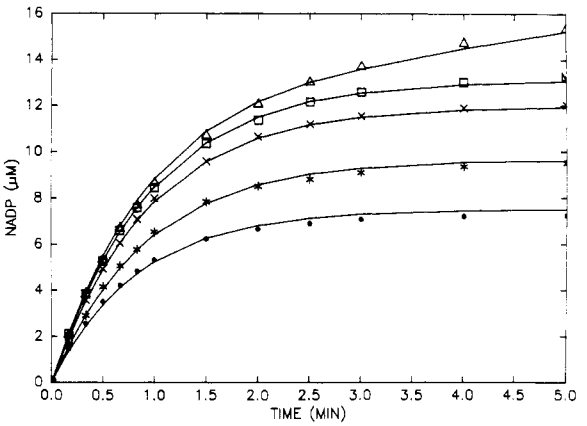


FIGURE 2: Progress curves in the presence of 0.5 mM EDTA and the absence of thiol reagents. Reaction mixtures contained buffer, NADPH (100 μM), EDTA (0.5 mM), and the following concentrations of Hg(II): (△) 18, (□) 24, (×) 36, (*) 72, and (●) 108 μM. Reactions were initiated by the addition of enzyme (10.3 nM). The continuous lines are computed curves, obtained as described in the text.

Au(III), which was noncompetitive. It was noted from these results that all of the inhibitory metal ions have in common a high affinity for thiols.

Progress Curves. The oxidation of NADPH by mercuric reductase under standard assay conditions was biphasic, with a rapid initial phase which was followed by a slower, linear phase. When 2-mercaptoethanol was removed from the assay mixture, the slower phase was eliminated, and the reaction rate rapidly leveled off to zero rate. In the absence of both 2-mercaptoethanol and EDTA there was no enzyme activity detected. The following studies were undertaken to clarify these initial observations.

Progress curves were obtained at various mercuric ion concentrations in the absence of thiol reagent and constant EDTA. As shown in Figure 2, there is a time-dependent decrease in the reaction rate which varies as a function of the mercuric ion concentration. At higher Hg(II) concentrations there is complete elimination of the reaction rate after only a few minutes, suggesting the formation of an irreversible enzyme–inhibitor complex. Similar curves were obtained when enzyme was preincubated with Hg(II) plus EDTA and the reaction initiated with NADPH, indicating that a reduced form of the enzyme is probably required for the formation of the irreversible inhibitory complex. Because of the difficulty of estimating initial velocities from the above curves, the data from each curve of Figure 2 and several other curves were fitted separately to eq 1, and the resulting values of v_0 were used to construct a reciprocal plot. This reciprocal plot, shown in Figure 3 (top), indicates substantial substrate inhibition by Hg(II) in the presence of EDTA. The initial velocities (v_0) were also plotted as a function of EDTA concentration at a fixed concentration of Hg(II) (Figure 3, bottom). The magnitude of the Hg(II)–EDTA stability constant (Sillen & Martell, 1964) would ensure that in the presence of an excess of EDTA all of the Hg(II) would be in the EDTA complex. As shown, there was no detectable reaction rate until the ratio of EDTA/Hg(II) = 1. At this point the initial velocity rose rapidly to its highest value and remained unchanged over a 10-fold increase in the EDTA/Hg(II) ratio. These results indicate that the role of EDTA is to complex free Hg(II) and it is the EDTA–Hg(II) complex which acts as the substrate in the absence of thiols.

The data are thus qualitatively consistent with the mechanism shown in Scheme I. Table III lists the values of the kinetic constants associated with this mechanism, which were determined by computer analysis of the progress curve data according to the procedure outlined under Data Analysis.

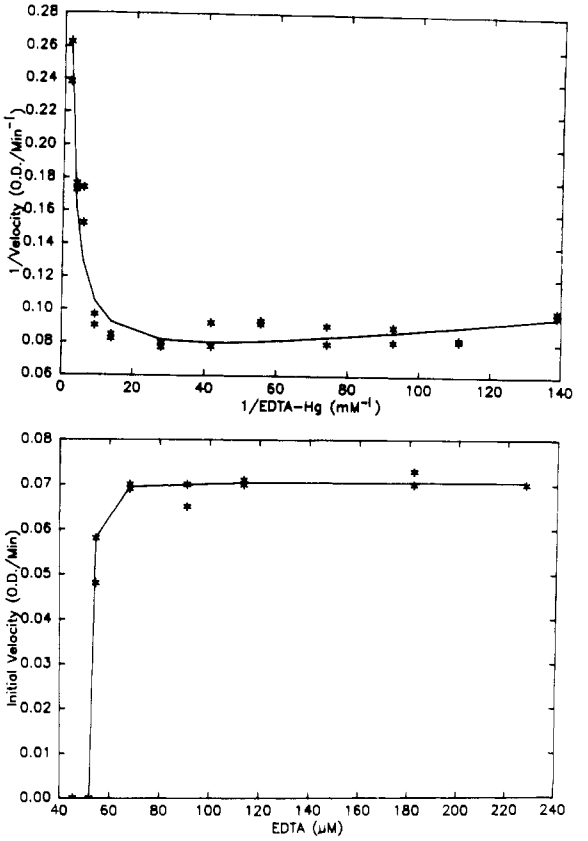


FIGURE 3: Inhibition of mercuric reductase by Hg–EDTA. (Top) Substrate inhibition by Hg–EDTA. The reaction velocity corresponds to v_0 of eq 1. (Bottom) Initial velocity (v_0) as a function of EDTA concentration. Hg(II) and enzyme were constant in each assay at 55 μM and 10.3 nM, respectively.

Scheme I

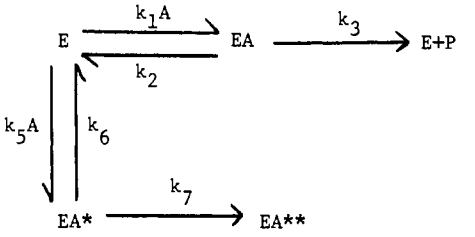


Table III: Kinetic Constants of Mercuric Reductase in the Presence of EDTA[a]

parameter	value	error[e]
$V/(E_t)$ (min^{-1})	1038	21
K_A (μM)	0.46	0.12
K_i (μM)	125	6
k_7 (min^{-1})	298	97
$K_{1/2}$ (SH) (mM)[b,c]	1.06	0.12
k_{rec} (min^{-1})[b,d]	0.09	0.02

[a] Progress curve data from Figure 2 were analyzed by fitting to the mechanism of Scheme I by using the STEPIT program. The concentrations of NADPH and enzyme were 100 μM and 10 nM, respectively. The error values are standard errors from overall fits. E in Scheme I represents reduced enzyme. [b] These values represent recovery of activity with RSH after inactivation with EDTA–Hg (Figure 4). [c] This value represents the concentration of RSH which yields half the maximal rate of activity recovery. [d] This value represents the maximal rate of activity recovery from extrapolation to infinite concentration of RSH. [e] The error values are standard errors from overall fits.

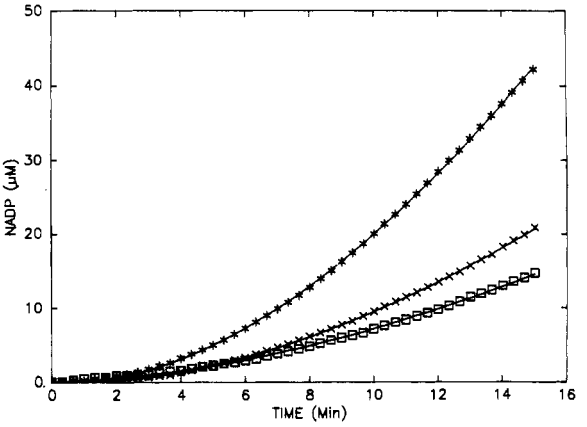


FIGURE 4: Progress curves for the recovery of the activity of mercuric reductase by addition of 2-mercaptoethanol. Enzyme was incubated for 15 min with 108 μM Hg(II), 100 μM NADPH, and 1.0 mM EDTA, after which reaction was initiated by the addition of (□) 2.8, (×) 5.4, or (*) 8.0 mM 2-mercaptoethanol. The continuous lines represent fitted values.

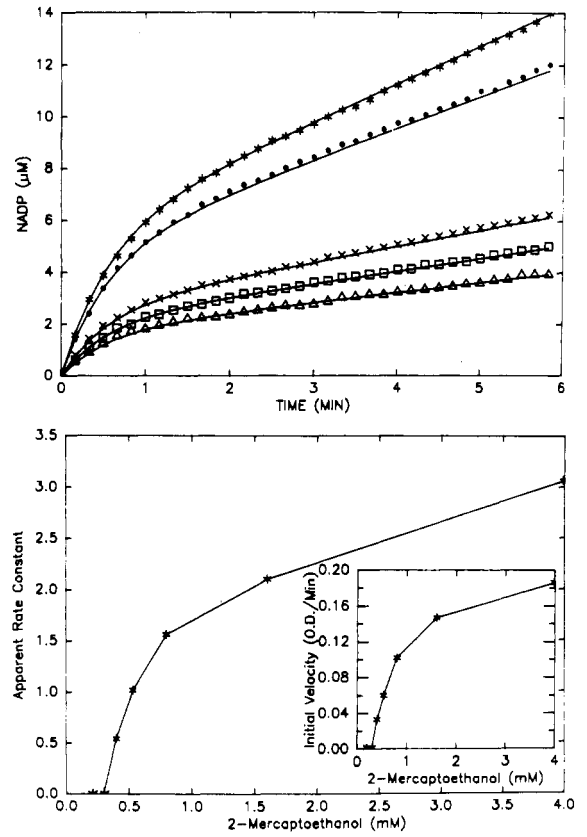


FIGURE 5: Biphasic kinetics of mercuric reductase in the presence of thiol reagents. (Top) Progress curves in the presence of 1 mM 2-mercaptoethanol and the absence of EDTA. Reaction mixtures contained buffer, NADPH(100 μM), 2-mercaptoethanol (1 mM), and the following concentrations of Hg(II): (*) 18, (●) 24, (×) 36, (□) 72, and (△) 108 μM. Reactions were initiated by the addition of enzyme. The continuous lines are computed curves, obtained as described in the text. (Bottom) Apparent rate constant (k') for the transition to less active enzyme as a function of thiol concentration. Hg(II) and enzyme were constant in each assay at 55 μM and 10 nM, respectively. [Bottom (inset)] Initial velocity (v_0) as a function of thiol concentration.

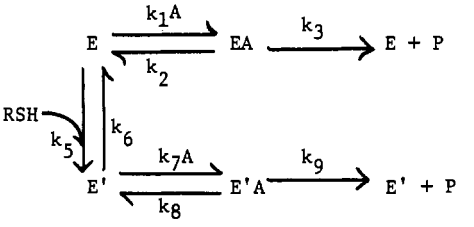
Although mercuric reductase is irreversibly inhibited by EDTA–Hg(II), enzyme activity can be slowly reclaimed by the addition of thiols (Figure 4), indicating the reaction can be made reversible. The kinetic constants associated with the thiol reactivation are included in Table III. It should be

Table IV: Kinetic Constants of Mercuric Reductase in the Presence of 2-Mercaptoethanol[a]

parameter	value	error[e]
$V/(E_t)$[b] (min^{-1})	1044	21
K_A (μM)[b]	53	2
$V'/(E_t)$[c] (min^{-1})	152	4
K'_A (μM)[c]	70	3
k'_s (min^{-1})[d]	1.8	0.3

[a] Progress curve data from Figure 5 (top) were analyzed by fitting to the mechanism of Scheme II by using the STEPIT program. The concentrations of NADPH and enzyme are the same as in Table III. E and E′ of Scheme II represent reduced enzyme. [b] These values represent constants associated with the E form in Scheme II. [c] These values represent constants associated with the E′ form in Scheme II. [d] This value represents the apparent first-order constant for the conversion of E to E′. k' is constant at a fixed concentration of 2-mercaptoethanol which was 1 mM in this experiment. [e] The error values are standard errors from overall fits.

Scheme II



pointed out that the interaction of thiols with the inhibited enzyme was complicated by other factors as described below.

The effect of thiols on mercuric reductase is markedly different from that of EDTA. When the enzyme is assayed in the absence of EDTA and a constant concentration of thiol reagent (2-mercaptoethanol), the progress curves are typically biphasic (Figure 5, top). The rapid kinetic phase was not oxygen dependent, since identical progress curves were obtained under anaerobic conditions. The slow transition from a rapid to a slower phase shown in these curves is characteristic of what Frieden (1979) refers to as hysteretic behavior. In general, three parameters can be obtained from each progress curve in the analysis of a slow transition: the initial velocity (v_0), the steady-state velocity (v_s), and the apparent rate constant for the transition between these two velocities (k'). In hysteretic enzyme systems, k' is expected to be dependent on the concentration of the effector ligand. When k' was determined for each of the curves in Figure 5 (top), it was found to be constant. However, k' was shown to increase significantly when Hg(II) was held constant and the concentration of 2-mercaptoethanol was increased (Figure 5, bottom). Finally, the initial fast phase of the progress curves can be eliminated by preincubating the enzyme with NADPH and 2-mercaptoethanol. When reactions are then initiated with Hg(II), only the slower, linear portion of the curves is observed. These results suggest that mercuric reductase exists in at least two separate forms and that thiol reagents effect the conversion of the enzyme into the less active of the two forms. A mechanism consistent with these observations is shown in Scheme II. Table IV lists the values of the kinetic constants determined from an analysis of the progress curve data in Figure 5 (top) as outlined under Data Analysis. It should be noted that it was not necessary to invoke cooperativity to fit the experimental results.

Spectroscopic Studies. The spectrum of oxidized mercuric reductase shown in Figure 6, curve A, is strikingly similar to that of glutathione reductase, as noted previously by Fox &

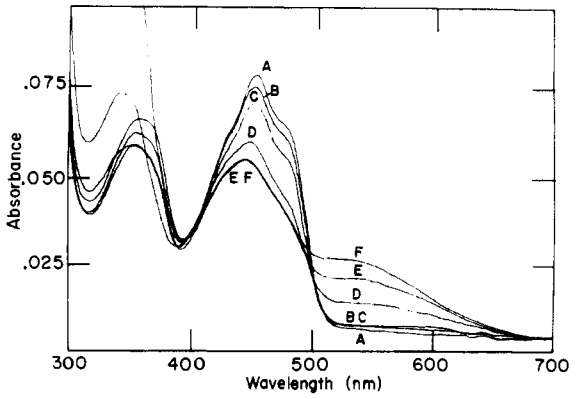


FIGURE 6: Anaerobic titration of mercuric reductase with NADPH. (A) 6.3 μM oxidized enzyme, (B) NADPH/FAD = 0.28, (C) NADPH/FAD = 0.76, (D) NADPH/FAD = 1.2, (E) NADPH/FAD = 2.5, and (F) NADPH/FAD = 4.1.

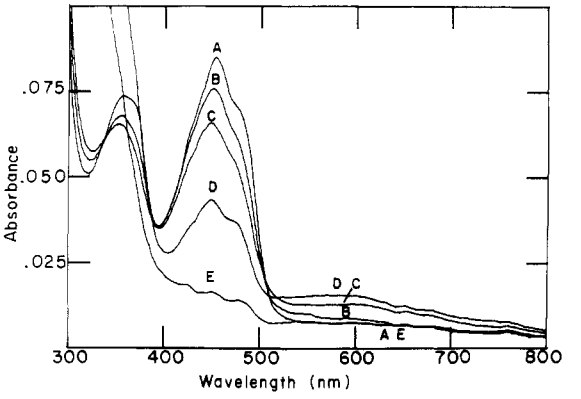


FIGURE 7: Anaerobic titration of mercuric reductase with NADPH in the presence of 0.1 M arsenite. (A) 6.8 μM oxidized enzyme, (B) NADPH/FAD = 1.1, (C) NADPH/FAD = 2.2, (D) NADPH/FAD = 4.1, and (E) same conditions as (D), except 8 h after the addition of NADase.

Walsh (1982). Furthermore, the anaerobic titration of the enzyme with NADPH could only eliminate about 33% of the characteristic absorption of FAD at 455 nm (Figure 6). It was also observed that as NADPH was increased, there appeared an absorption at 540 nm which indicated the formation of a stable enzyme intermediate which has been attributed to a charge-transfer complex between a thiolate anion and oxidized FAD (Williams, 1976). When the titration was repeated in the presence of 0.1 M arsenite, the flavin appeared to be almost completely reoxidized a few minutes after the addition of 1 equiv of NADPH (Figure 7). Further additions of NADPH resulted in a marked reduction of the absorbance at both 455 and 540 nm and an increase in the absorbance at longer wavelengths. The long wavelength absorbance has been previously observed in similar enzymes and has been attributed to a charge-transfer complex between NADP and $FADH_2$ (Williams, 1976). This was verified in our case by the complete loss of the long wavelength absorption after the addition of NADase, which specifically hydrolyzes the oxidized form of the pyridine nucleotides. As shown in Figure 7, curve E, removal of the enzyme-bound NADP also allowed the complete reduction of FAD. Apparently, the NADP–enzyme complex interferes with the reduction of the enzyme by NADPH.

Thiol Titrations. The spectroscopic studies suggested mechanistic similarities between mercuric reductase and a group of flavoproteins. These enzymes, such as glutathione reductase, all contain FAD and an active site disulfide which undergoes reduction and oxidation during the catalytic cycle

Table V: Determination of Thiols in Oxidized and Reduced Mercuric Reductase[a]

enzyme	SH/FAD	error[c]
oxidized	3.2	0.2
reduced[b]	5.1	0.3

[a] Thiols were quantitated with 0.5 mM 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959) after the enzyme was denatured in 0.1 M Tris, pH 8.0, and 8 M urea, at 60 °C for 15 min. [b] The enzyme was reduced with 0.1 mM NADPH before denaturation and determination of thiols. [c] The error values represent the SD of three separate determinations.

(Williams, 1976). Table V shows the results of an enzyme thiol titration with 5,5′-dithiobis(2-nitrobenzoate). Similar to the results reported by Fox & Walsh (1982), we found two more thiols in the enzyme after it had been reduced by NADPH and denatured prior to thiol determination. These results confirm the presence of a reducible active site disulfide. Interestingly, during the course of these studies we determined that 5,5′-dithiobis(2-nitrobenzoate) could be catalytically reduced to the thiolate anion by mercuric reductase in the presence of NADPH. This substrate was determined to have a K_m of 3.3 ± 0.3 mM and a V_{max} value, 1% that observed with Hg(II). Other disulfide substrates tested, such as oxidized glutathione, were found to be inactive. These results demonstrate a very close relationship between mercuric reductase and other redox-active, disulfide-containing flavoproteins.

Discussion

Mercuric reductase from *E. coli* KP245 is very similar to the enzyme isolated previously from other sources. However, there was a significant difference in the amount of enzyme produced by this strain and the *P. aeruginosa* strain described by Fox & Walsh (1982). Since the *mer* genes in both strains have been genetically amplified, this may suggest substantial differences in gene regulation. Previous reports indicated that the enzyme exists in a heavy form (M_r 62 000) which is converted into a lighter form (M_r 56 000) by some unknown mechanism (Schottel, 1978; Fox & Walsh, 1982). In our preparations, only a protein of monomer M_r 56 000 could be detected. Jackson & Summers (1982) have recently shown that five Hg(II)-inducible polypeptides are synthesized from pRR130 in minicell systems. The two largest polypeptides (M_r 69 000 and 66 000) were shown to be immunologically related to the purified reductase. The apparent molecular weight disparity between these proteins and that of our purified enzyme is unexplained but does suggest significant posttranslational modification. Mercuric reductase is very specific for mercuric ions, reducing none of the metal ions shown in Table I. Even Ag(I), with a favorable redox potential, was not reduced although it appeared to bind well to the enzyme. These results suggest the enzyme may only operate through a two-electron cycle with the one-electron-reduced enzyme being inactive. The noncompetitive inhibition by Au(III) requires an alternate explanation. It would appear that this compound is not reduced because it does not bind to the proper site on the enzyme.

The spectroscopic data and the thiol titration (Table V) clearly identify mercuric reductase as belonging to the pyridine nucleotide–disulfide reductase class of flavoproteins. As shown in Figure 6, addition of reducing equivalents (NADPH) to mercuric reductase produces a spectrum similar to the EH_2 form of glutathione reductase and lipoamide dehydrogenase. In the presence of arsenite (Figure 7), which complexes quite specifically with vicinal dithiols, the EH_2 form of mercuric reductase is not stable and full reduction to EH_4 results.

Finally, the sulfhydryl titration demonstrated that two enzyme thiols became accessible upon reduction with NADPH. These are all characteristic features of the disulfide reductase class of flavoproteins. Since both glutathione reductase (Icen, 1967) and mercuric reductase are able to accept 5,5′-dithiobis(2-nitrobenzoate) as a substrate, the relationship between these enzymes must be very close. From these results, the mechanism of mercuric reductase probably involves the transfer of electrons from NADPH to FAD, then to the active site disulfide, and eventually to the chelated Hg(II). Fox & Walsh (1982) have recently proposed a similar mechanism but have raised questions as to whether the reductase cycles between E and EH_2 or EH_2 and EH_4 during turnover.

EDTA and 2-mercaptoethanol have a marked effect on the kinetics of mercuric reductase. In the absence of both reagents there is no enzyme activity. It thus appears that free Hg(II) is not a substrate. Rather, it must act as a tightly bound inhibitor, since low concentrations of Hg(II) (>1 μM) are enough to prevent the catalytic reduction of 5,5′-dithiobis(2-nitrobenzoate) in the absence of EDTA and thiol reagents. Figure 2 shows that in the presence of EDTA, Hg(II) can act as a substrate which undergoes multiple turnovers before complete enzyme inactivation. As shown in Figure 3 (bottom), it would appear the effect of EDTA is to complex the free Hg(II), eliminating the inhibitory free ion. In support of this conclusion, we found that other chelating agents which bind mercury less effectively were unsuccessful at preventing immediate enzyme inactivation. This point, together with the evidence for a rapidly formed enzyme–substrate, inhibitory complex (Figure 3, top), suggested the mechanism for the EDTA–Hg(II) results outlined in Scheme I. The mechanism involves the rapid reaction of the complexed Hg(II) with enzyme to form a productive (EA) or a nonproductive (EA*) enzyme intermediate. The EA* complex then undergoes a slow, irreversible reaction to form EA**. Analysis of the progress curve data according to this mechanism yielded estimates of the parameters in Scheme I (Table I) with low standard errors. Thus the data appear both qualitatively and quantitatively in agreement with the mechanism of Scheme I.

From the values in Table I it is possible to calculate the number of catalytic events before inactivation according to the relationship r = turnover/k_7 = 3.5. This value is very low, indicating that EDTA is not very effective at preventing enzyme inactivation. From this result, a probable mechanism of enzyme inactivation would involve ligand exchange between the EDTA and thiols on the enzyme resulting in Hg(II) bound incorrectly in the active site. This type of exchange is known to occur quite rapidly and would favor formation of the thiol–Hg(II) complex (Rabenstein & Fairhurst, 1975). This mechanism is supported by the data in Figure 4 which shows that EDTA-inactivated enzyme can be reactivated by the addition of free thiols. The added thiols would be expected to compete as ligands for the enzyme-bound Hg(II), thus liberating it from the enzyme.

Besides liberating enzyme-bound mercury, thiol reagents also have a direct effect on the enzyme. As shown in Figure 5, it is 2-mercaptoethanol, not Hg(II), which has a significant effect on the apparent rate of the transition to the slower steady-state reaction rate. This type of kinetic behavior has been termed hysteretic by Frieden (1979). Enzymes that show hysteretic behavior are defined as those that respond slowly to a change in an effector ligand concentration. Several models have been proposed to account for hysteretic enzymes. As discussed by Frieden, most mechanisms for hysteretic behavior assume at least two forms of the enzyme which differ in their kinetic properties. Assuming the most simple model shown in Scheme II, we were able to satisfactorily determine the kinetic parameters for each of the two forms of the enzyme (Table IV) from the data in Figure 5 (top). This analysis did not require inclusion of cooperative parameters although they cannot be totally eliminated from the model based solely on these experiments. Thus, from our results it appears that thiol reagents react slowly with mercuric reductase to bring about a transition of the enzyme to a less active form, and it is the formation of this enzyme form which is responsible for the observed biphasic kinetics of mercuric reductase.

From the values in Table IV we are able to make a comparison between the two forms of the enzyme, E and E′. It can be seen that there is only a very small difference between the K_m values, but a significant difference in the values for V_{max} which is reflected in even a slightly greater difference in the V/K values. These results suggest that binding of substrate has not been altered in E′. But the large differences in V_{max} indicate that product release or catalysis has become much slower. The large differences in the V/K values would argue in favor of a substantial decrease in the rate of the catalytic step. It was noted that the V_{max} values of E determined in the presence of thiols or EDTA (Table III) agree within experimental error as expected. However, the K_m values differ by a factor greater than 100, indicating that Hg(II)–EDTA binds better to the enzyme than RS–Hg–SR. This may be due to the ligand-exchange process discussed above. An intermediate K_m value (14 ± 2 μM), which agrees more favorably with previously reported values (Fox & Walsh, 1982; Schottel, 1978), was obtained from initial rate measurements in the presence of EDTA and 2-mercaptoethanol together.

Neet & Ainslie (1980) have discussed some molecular aspects of the slow conformational changes associated with hysteretic enzymes. In general, a slow conformational change in a protein requires a relatively high energy barrier when compared to other more rapid steps. This barrier may be represented by such things as the making or breaking of many bonds simultaneously, charge repulsion or steric hindrance in the course of the movement, or unfavorable rotation about a single bond. In the case of mercuric reductase, the slow conformational change is caused by reaction of the enzyme with thiols. On the basis of the known chemistry of thiols, it might be expected that the slow conformational change involves disulfide reduction, either at the active site or elsewhere on the protein molecule. The slow conformational changes accompanying the reduction of a disulfide could be due to any of the processes described above. At present it is unclear which mechanism might be involved. Studies are currently in progress to more fully characterize the effect of thiols on mercuric reductase.

Acknowledgments

We thank Dr. Simon Silver of Washington University for useful discussions of this work. We also thank Dr. Arnold Weinrib and John Fowble for their assistance in the computerization of the spectrophotometer.

Registry No. Cd, 7440-43-9; Ag, 7440-22-4; Cu, 7440-50-8; Au, 7440-57-5; cysteine, 52-90-4; EDTA, 60-00-4; 2-mercaptoethanol, 60-24-2; mercuric reductase, 67880-93-7.

References

Cha, S. (1976) *Biochem. Pharmacol. 25*, 2695–2702.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1–29.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 500–513.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70-77.
Fox, B., & Walsh, C. F. (1982) *J. Biol. Chem. 257*, 2498-2503.
Frieden, C. (1979) *Annu. Rev. Biochem. 48*, 471-489.
Furukawa, K., & Tonomura, K. (1972) *Agric. Biol. Chem. 36*, 217-226.
Icen, A. (1967) *Scand. J. Clin. Lab. Invest. 20*, 96-99.
Izaki, K., Tashiro, Y., & Funaba, F. (1974) *J. Biochem. (Tokyo) 75*, 591-599.
Jackson, W. J., & Summers, A. O. (1982) *J. Bacteriol. 149*, 479-487.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Meites, L., & Meites, T. (1948) *Anal. Chem. 20*, 984-985.
Nakahara, H., Kinscherf, T. G., Silver, S., Miki, F., Easton, A. M., & Rownd, R. H. (1979) *J. Bacteriol. 138*, 284-287.
Neet, K. E., & Ainslie, G. R., Jr. (1980) *Methods Enzymol. 64*, 192-226.
Rabenstein, D. L., & Fairhurst, M. F. (1975) *J. Am. Chem. Soc. 97*, 2086-2092.
Ryan, L. D., & Vestling, C. S. (1974) *Arch. Biochem. Biophys. 160*, 279-284.
Schottel, J. S. (1978) *J. Biol. Chem. 253*, 4341-4349.
Sillen, L. G., & Martel, A. E. (1964) *Spec. Publ.—Chem. Soc. No. 17*, 637.
Summers, A. O., & Silver, S. (1978) *Annu. Rev. Microbiol. 32*, 637-672.
Tezuka, T., & Tonomura, K. (1976) *J. Biochem. (Tokyo) 80*, 79-87.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406-4412.
Williams, C. H. (1976) *Enzymes, 3rd Ed. 13*, 89-173.
Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437-467.
Williams, J. W., Duggleby, R. G., Cutler, R., & Morrison, J. F. (1980) *Biochem. Pharmacol. 29*, 589-595.

Fate of Oxygen during Ferritin Iron Incorporation†

Dennis E. Mayer, Jeffrey S. Rohrer, Dale A. Schoeller, and Daniel C. Harris*

ABSTRACT: The deposition of Fe(II) into ferritin using $^{18}O_2$ as the oxidant was investigated. Only 3-4% of the oxygen atoms in the FeOOH core of ferritin were derived from the oxidant. This was true whether large (1200-1900 Fe/molecule) or small (220-240 Fe/molecule) amounts of iron were added to apoferritin or when iron was added to ferritin already containing 1000 Fe/molecule. Experiments using $H_2{}^{18}O$ in the solvent showed that nearly all of the oxygen atoms in the core were derived from solvent. The stoichiometry of the reaction was close to 2 Fe(II)/O_2, instead of the expected value of 4 Fe(II)/O_2. Reactions of $^{18}O_2$ with Fe(II) in the absence of apoferritin had a similar ^{18}O distribution and stoichiometry. Our results are most consistent with the crystal growth model of ferritin iron deposition.

The iron-storage protein, ferritin, is found all through the animal and plant kingdoms (Crichton, 1973; Aisen & Litowsky, 1980; Harrison, 1977). It has a hollow spherical protein shell with a molecular weight near 450 000. Inside is a core containing from 0 to about 4300 iron atoms in the form FeOOH, with some associated phosphate. The protein shell has an inner diameter of 7-8 nm and an outer diameter of 12-13 nm. It is comprised of 24 subunits arranged with 432 symmetry so that there are six channels piercing the shell along Cartesian axes (Banyard et al., 1978). Each channel has a diameter of about 1 nm, allowing small molecules to pass through to the core.

Although the core stores Fe(III), it is generally believed that mobilization and deposition involve Fe(II). While thioglycolic acid is most commonly used in the laboratory to reduce ferritin to apoferritin, the most rapidly acting reductants are flavins (Sirivech et al., 1974; Jones et al., 1978). In the presence of oxygen and Cu^{2+}, ascorbic acid may be an important physiologic reductant (Bienfait & Van Den Briel, 1980). In contrast, Fe(III) mobilization by chelating agents is not as fast as reductive removal (Crichton et al., 1980a; Pape et al., 1968; Tufano et al., 1981). Although apoferritin rapidly accumulates thousands of iron atoms per molecule in a solution containing Fe(II) and O_2, Fe(III) is taken up slowly and to a much smaller extent (Treffry & Harrison 1979).

When a solution of apoferritin and Fe(II) is exposed to oxygen, iron accumulates inside the protein shell in the form FeOOH (Macara et al., 1972). The simplest equation that can be written for this process leads us to expect that 4 mol of Fe(II) will react with 1 mol of O_2:

$$4Fe^{2+} + O_2 + 6H_2O \rightarrow 4FeOOH + 8H^+$$

The present study was undertaken to learn the fate of the molecular oxygen involved in Fe(II) uptake by ferritin.

Experimental Procedures

Ferritin. Horse spleen ferritin was purchases from Miles Laboratories (6 times crystallized, Cd removed) or from Sigma Chemical Co. (Type I). It was either used as received or purified by passage through an ascending column (2.6 × 80 cm) of Sepharose 6B eluted with 0.1 M Tris,[1] pH 7.0 (Niitsu & Listowsky, 1973). Apoprotein was prepared by dialysis against three portions of 0.1 M thioglycolic acid–0.1 M sodium acetate at its own pH (4.5) or against the same reagent at pH 5.0 if the protein precipitated. (At pH 5.0 the ferritin redissolves.) The reduction was followed by extensive dialysis

† From the Department of Chemistry, Franklin and Marshall College, Lancaster, Pennsylvania 17604 (D.E.M., J.S.R., and D.C.H.), and the Department of Medicine, University of Chicago, Chicago, Illinois 60637 (D.A.S.). *Received August 12, 1982.* This research was supported by grants from the National Institutes of Health (HL 28685 and AM 26678) and a Cottrel College Science grant from the Research Corporation.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; STP, standard temperature (0 °C) and pressure (1 atm).

0006-2960/83/0422-0876$01.50/0