Kinetic Study of the Dissolution of $Fe_4S_4^{2-}$-Cluster Core Ions of Ferredoxins and High Potential Iron Protein†

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ABSTRACT: The pH dependence of the dissolution of the $Fe_4S_4^{2-}$-cluster core ions of *Chromatium vinosum* high potential iron protein (HIPIP) and five ferredoxins has been determined. Between pH 6 and 11, the rate constants for dissolution of HIPIP are less than 10^{-8} s^{-1}. Below pH 6 and above pH 11 the dissolution of the $Fe_4S_4^{2-}$ cluster of HIPIP is second-order in hydrogen ion activity and second-order in hydroxide ion, respectively. The pH dependence of the dissolution of the ferredoxin $Fe_4S_4^{2-}$ clusters is more complex but each of the five ferredoxins obey the same rate equation. Between pH 6 and 9, the pseudo-first-order dissolution rate constants (k_{obsd}) are pH independent and, above pH 9, k_{obsd}'s are second-order in hydroxide ion concentration. Below pH 3 the values of k_{obsd} change from second-order dependence upon hydrogen ion activity to values independent of pH. At pHs less than 1, k_{obsd} again becomes dependent upon the second power of the hydrogen ion activity. In the acid range, the log k_{obsd} vs. pH profiles for dissolution of the $Fe_4S_4^{2-}$ clusters of the ferredoxins follow the same rate law as that for hydrolysis of synthetic $Fe_4S_4(SR)_4^{2-}$ cluster ions. Based on the analogy of the kinetics of the ferredoxins and model compounds, it is concluded that, at acid pHs, the $Fe_4S_4(S\text{-}Cys)_4^{2-}$ clusters of the ferredoxins undergo dissolution by hydrolysis of the $Fe_4S_4^{2-}$-cluster core ions, while at neutral and basic pH values dissolution is related to initial ligand exchange and possible extrusion of the $Fe_4S_4^{2-}$-cluster core ion which is then hydrolyzed. Kinetically competent mechanistic schemes are provided. The log k_{obsd} vs. pH profile for dissolution of *Bacillus polymyxa* ferredoxin, which contains only one $Fe_4S_4^{2-}$ cluster, follows the same rate law as do the ferredoxins of *Chromatium vinosum, Clostridium acidi-urici,* and *Cl. pasteurianum* which contain two $Fe_4S_4^{2-}$ clusters. This finding is interpreted to indicate that for the 8Fe-8S ferredoxins the rate and acid-base equilibrium constants are very similar for both $Fe_4S_4^{2-}$-cluster core ions. No correlation between midpoint redox potentials of ferredoxins and their hydrolytic reactivities was detected.

Ferredoxins and (presumably) high potential iron proteins (HIPIP) are involved as electron carrier proteins in various biological redox reactions (Mortenson and Nakos, 1973). They are characterized by one or more acid-labile Fe_2S_2- or Fe_4S_4-cluster core ions which are complexed to cysteine sulfurs of the apoprotein. In the process of electron transfer, the oxidation states of clusters in 4Fe-4S and 8Fe-8S ferredoxins alternate between $Fe_4S_4^{2-}$ and $Fe_4S_4^{3-}$, while the cluster in HIPIP alternates between $Fe_4S_4^{-}$ and $Fe_4S_4^{2-}$ (Carter et al., 1972). With the identification and resolution of the structures of the "active site" of various iron-sulfur proteins (Carter et al., 1972), questions concerning the chemistry of the protein-complexed iron-sulfur clusters become important.

Stabilizing effects of the protein upon the iron-sulfur-cluster core ion can be viewed in terms of chelation and shielding. The chemical reactivity of protein-bound iron-sulfur clusters toward various solute species can be employed as a measure of the accessibility of the Fe_2S_2- and Fe_4S_4-cluster core ions. A number of investigations of the comparative reactivity of selected iron-sulfur proteins with various reagents have been reported. These include: the reaction of triphenylphosphine with adrenal, spinach, and *Clostridium pasteurianum* ferredoxins (Manabe et al., 1976); water and oxygen with *Cl. acidi-urici* ferredoxin and its variously altered amino acid derivatives (Lode et al., 1976a; Hong and Rabinowitz, 1970a); *p*-chloromercuribenzoate with spinach, *Cl. pasteurianum,* and *Azotobacter vinlandii* ferredoxins (Yoch and Arnon, 1972); and others (Rawlings et al., 1976; Sweeney et al., 1974; Cammack, 1973; Petering et al., 1971; Flatmark and Dus, 1969; Malkin and Rabinowitz, 1967; Malkin and Rabinowitz, 1966). These investigations have not examined the pH dependence of the stability of the iron-sulfur-cluster core ions. Knowing the dependence of the rate of dissolution of the protein-bound iron-sulfur-cluster core ion on the concentration of H_3O^+ and HO^- is most important in the design of comparative kinetic studies. In addition, on the basis of microscopic reversibility, a knowledge of the rate law for dissolution of the cluster core ions should be of use in understanding the mechanism of synthesis of the protein-bound iron-sulfur clusters. In this study, the log k_{rate} vs. pH profiles for the dissolution of the $Fe_4S_4^{2-}$-cluster core ions of five ferredoxins and *Chromatium vinosum* HIPIP are reported, cluster site reactivities are compared, and mechanisms for the reaction of solute species with the $Fe_4S_4^{2-}$-cluster core ions of these proteins are discussed.

Experimental Section

Chromatium vinosum ferredoxin and high potential iron protein (HIPIP[1]) (Bartsch, 1971), and the ferredoxins from *Bacillus polymyxa* (type II) (Stombaugh et al., 1973) and *Cl. pasteurianum* (Lovenberg and Sobel, 1965) were prepared by published methods. *Cl. acidi-urici* ferredoxin (Buchanan et al., 1963) and a derivative where the normal tyrosine-2 residue is replaced by leucine, *acidi-urici* [Leu][2] (Lode et al., 1974), were reconstituted from the apoenzymes by the method of Hong and Rabinowitz (1970b). All ferredoxin samples employed in this study were provided as gifts by the contributors listed in the Acknowledgment.

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[1] Abbreviations used: HIPIP, high potential iron protein; Tris, tris(hydroxymethyl)aminomethane.

Ferredoxins employed contained cluster ion cores in the oxidized form, e.g., $Fe_4S_4^{2-}$. The HIPIP employed was in the reduced form, containing a $Fe_4S_4^{2-}$-cluster core ion. Purity indexes of the various iron-sulfur proteins were: *Chromatium vinosum* ferredoxin, A_{278}/A_{390} = 2.09; *C. vinosum* HIPIP, A_{283}/A_{388} = 2.56; *Bacillus polymyxa* type II, A_{395}/A_{280} = 0.67; *Cl. pasteurianum*, A_{390}/A_{280} = 0.80; *Cl. acidi-urici*, A_{390}/A_{280} = 0.78; and *Cl. acidi-urici* [Leu]^2, A_{390}/A_{280} = 0.78. Absorbance ratios were measured in 0.1 M KCl on a Cary Model 118 spectrophotometer.

Rapid reaction rates were obtained using a Durrum Model D-110 stopped-flow spectrophotometer (under a N_2 atmosphere) with a Biomation Model 805 waveform recorder. Slow reactions were followed using Thunberg cuvettes and a Gilford Model 2000 spectrophotometer. Temperature was maintained at 30.0 °C.

Measurements were performed anaerobically under pseudo-first-order conditions ([buffer] > 100[iron-sulfur protein]). The ionic strength employed at all pHs was 1.0 (except at HCl concentrations greater than 1.0 M) in terms of buffer species and KCl. HCl was employed as a buffer between $H_0 = -1.10$ and pH 2.50. Formate was employed between pH 2.45 and pH 3.20, and acetate was employed between pH 3.65 and pH 5.60. For reactions in the basic region, Tris was employed between pH 8.10 and 9.10. Carbonate was employed from pH 9.5 to pH 10.4, with phosphate being used between pH 11.0 and pH 12.90. KOH was employed to buffer at pH 13.70.

Kinetics of $Fe_4S_4^{2-}$-cluster core ion dissolution were monitored by observing the loss in absorbance of the Fe_4S_4 chromophore at 390 nm. Rate constants for reactions which were followed to completion were calculated via a linear least-squares first-order program. First-order rate constants were calculated for slow reactions from initial slopes. Initial slope rate constants were also calculated for reactions at pHs within ~1 pH unit of the p*I* of the proteins due to their precipitation at these pHs.

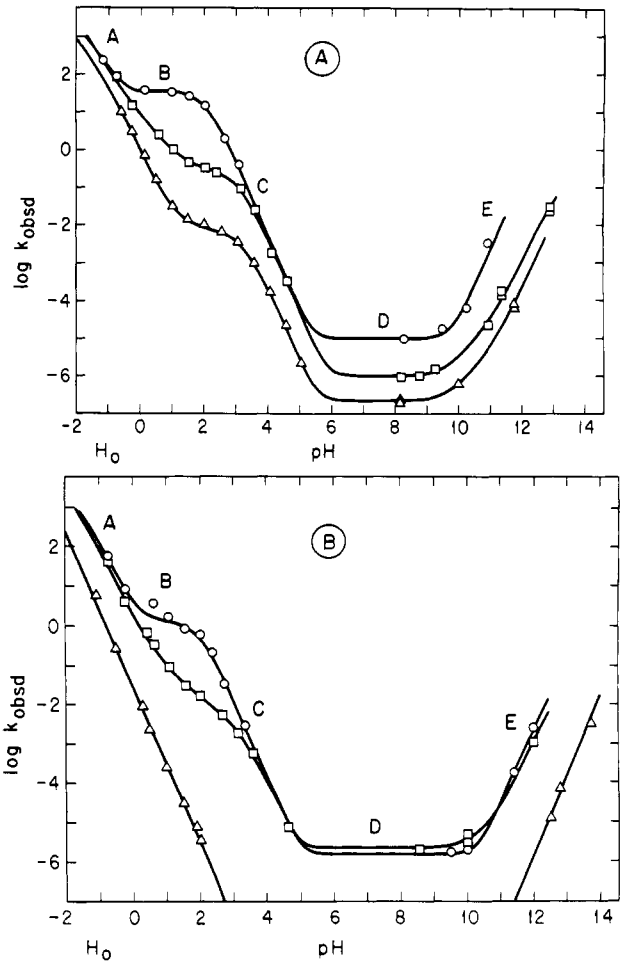


FIGURE 1: Log k_{obsd} vs. pH-rate profiles for the dissolution of Fe_4S_4 clusters in various ferredoxins. (A) (○) *Bacillus polymyxa* (type II); (□) *Clostridium acidi-urici* [Leu]^2; (△) *Clostridium acidi-urici*. (B) (○) *Chromatium vinosum;* (□) *Clostridium pasteurianum;* (△) *Chromatium* HIPIP (values of k_{obsd} are in s^{-1}).

Results

At constant pH the disappearance of absorbance of the $Fe_4S_4^{2-}$-cluster core ions (A_{390}) of the various ferredoxins and HIPIP were found to follow the first-order rate law. The dependence of the first-order rate constants (k_{obsd}) upon pH is presented in the form of log k_{obsd} vs. pH profiles in Figures 1A and 1B. The smooth lines drawn through the data points were computer generated from eq 1

$$k_{obsd} = \underbrace{\frac{(Aa_H^2 + Ba_H + C)a_H^2}{Da_H^3 + Ea_H^2 + Fa_H + G}}_{\text{(I)}} + \underbrace{\frac{H + I(K_w/a_H) + J(K_w/a_H)^2}{K + L(K_w/a_H)}}_{\text{(II)}} \quad (1)$$

where K_w is the autoprotolysis constant of water at 30 °C and a_H is the hydrogen ion activity determined at the glass electrode. The constants A through L are empirical and possess the required values to fit eq 1 to the various profiles. The relative reactivities in the acid region (at the plateau region B of Figures 1A and 1B) and at neutral and basic pH values are provided in Table I. Relative stabilities between *Cl. acidi-urici* and *acidi-urici* [Leu]^2 ferredoxins obtained from the data of this study may be compared with the relative stabilities determined previously (Lode et al., 1976a). In the previous study, the native ferredoxin was found to have a biological activity half-life of 185 days under anaerobic conditions, pH 7.4, 0.1 M Tris, 4 °C. The leucine-2 derivative ferredoxin exhibited a half-life of 13 days. In comparison, half-lives for cluster hydrolysis determined in the present study (A_{390}) were 37 and 8 days for the two respective proteins under anaerobic conditions, pH 8.0, 0.1 M Tris, 30 °C. From this data it may be surmised that the influence of temperature upon the destruction of the $Fe_4S_4^{2-}$-cluster core ion of the native *acidi* ferredoxin ($t_{1/2}$(4 °C)/$t_{1/2}$(30 °C) = 5) is somewhat larger than

TABLE I: Relative Reactivities of Fe_4S_4 Ferredoxins.[a]

Protein	Relative rates		
	pH 2	pH 9	pH 12
B. polymyxa	883	4.50	56.2
C. vinosum	35.1	0.660	2.00
Cl. acidi-urici [Leu]^2	19.8	0.446	1.00
Cl. pasteurianum	1.00	1.00	1.00
Cl. acidi-urici	0.625	0.092	0.141
C. vinosum HIPIP	1.57×10^{-4}		1.26×10^{-3}

[a] The absolute values of the rate constants for *Cl. pasteurianum* hydrolysis are 1.60×10^{-2} s^{-1} at pH 2, 2.09×10^{-6} s^{-1} at pH 9, and 1.32×10^{-3} s^{-1} at pH 12.

for the modified ferredoxin (1.65) which, as shown, is less stable.

Neither general acid nor general base catalysis of $Fe_4S_4^{2-}$-cluster core ion hydrolysis was found to occur. At pH 2.5 change of buffer from HCl to formate showed no effect on reaction rate. At pHs 10 and 12 changes in carbonate and phosphate buffer concentrations from 0.1 M (μ = 1.0) to 0.4 M (μ = 1.3) also showed no change in the observed rates.

Discussion

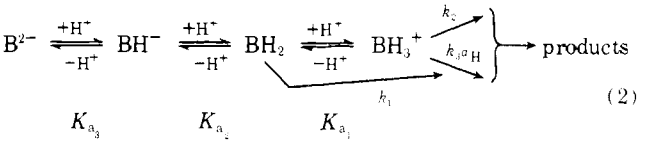
Differences in reactivities between the ferredoxins employed in this study can be rationalized in terms of differences in chelation geometry of the Fe_4S_4-cluster core ions and the four cysteine sulfhydryls by which the proteins bind the clusters, or in terms of different accessibilities of the clusters to solute reactant species. The role played by the protein tertiary structure in regulating the reactivity of $Fe_4S_4^{2-}$-cluster core ions toward various chemical agents is well documented. These observations include the greatly increased rates of reaction, on partial denaturation, of the $Fe_4S_4^{2-}$-cluster ion of *C. vinosum* HIPIP to dithionite (Cammack, 1973) and *Cl. acidi-urici* ferredoxin to chelating agents and oxygen (Malkin and Rabinowitz, 1967). X-ray crystallographic studies have established that the $Fe_4S_4^{2-}$-cluster core ion of HIPIP is protected from external medium by a pleated sheet covering (Carter et al., 1974a,b). This finding provides an explanation for observations such as the much slower rate of reaction of *C. vinosum* HIPIP as compared with *Cl. pasteurianum* ferredoxin with *p*-chloromercuribenzoate (Flatmark and Dus, 1969; Yoch and Arnon, 1972), and the low reactivity of HIPIP toward $Fe(EDTA)^{2-}$ where electron transfer occurs over a distance of $\geq$3.5 Å (Rawlings et al., 1976). The large change in hydrolytic stability of the $Fe_4S_4^{2-}$ clusters of *Cl. acidi-urici* on substitution of Tyr-2 by Leu provides a most appropriate demonstration of the role of protein structure in the accessibility and reactivity of the $Fe_4S_4^{2-}$-cluster core ions (Lode et al., 1976a).

The logs of first-order rate constants for dissolution of the $Fe_4S_4^{2-}$-cluster core ions of the proteins studied herein vs. pH values at which the rate constants were determined are provided in Figures 1A and 1B. Relative rate constants (relative to *Cl. pasteurianum*) for $Fe_4S_4^{2-}$-cluster disappearance at acid and basic pH are shown in Table I. Examination of this table reveals that stabilities of the $Fe_4S_4^{2-}$ clusters follows the order: acid pH, *C. vinosum* HIPIP $\gg$ *Cl. acidi-urici* $\cong$ *Cl. pasteurianum* > *Cl. acidi-urici* $[Leu]^2 \cong$ *C. vinosum* > *Bacillus* polymyxa type II; basic pH, *C. vinosum* HIPIP $\gg$ *Cl. acidi-urici* > *Cl. acidi-urici* $[Leu]^2 \simeq$ *Cl. pasteurianum* $\simeq$ *C. vinosum* > *B. polymyxa* type II. Therefore, in the pH range of -1 to 13, the least reactive $Fe_4S_4^{2-}$-cluster core ions are found in *C. vinosum* HIPIP and the most reactive in *B. polymyxa* type II ferredoxin.

The pH-log k_{obsd} profiles for $Fe_4S_4^{2-}$-cluster core ion dissolution of the various proteins are rather remarkable. The simplest is that for *C. vinosum* HIPIP. In the acid pH range, cluster dissolution is found to be strictly second-order in hydrogen ion activity to $H_0 = -1$ (i.e., slope of plot = -2). At basic pH values, the rate of disappearance of the $Fe_4S_4^{2-}$ cluster of HIPIP is second-order in hydroxide ion. At pH values between 3 and 11, HIPIP exhibits exceptional inertness to lyate species.

The pH-log k_{obsd} profile for the various ferredoxins may be divided into regions A, B, C, D, and E. In regions A and C dissolution of the $Fe_4S_4^{2-}$-cluster core ions are second-order in hydrogen ion concentration while in region B and D the rate constants are independent of acid and base. In region E, values of k_{obsd} are found to be dependent upon the second power of hydroxide ion concentration. The changes in dependence of k_{obsd} from zero-order to second-order in $[H_3O^+]$ and $[HO^-]$ are most simply ascribed to multiple acid–base equilibria. Thus, regions C and B can be explained by diprotonation of the $Fe_4S_4(S\text{-}Cys)_4^{2-}$ moieties ($pK_{a_3} > pK_{a_2}$) to provide $Fe_4S_4(S\text{-}Cys)_4H_2$ which undergoes dissolution at a log k_{rate} equivalent to the log k_{obsd} at region B. Region A can be rationalized by assuming that the $Fe_4S_4(S\text{-}Cys)_4H_2$ core undergoes an additional equilibrium protonation to yield $Fe_4S_4(S\text{-}Cys)_4H_3^+$ which undergoes dissolution by rate-controlling protonation, etc. Of considerable interest is the finding that the log k_{obsd} vs. pH profile for *B. polymyxa* type II ferredoxin is of the same form as that exhibited by the other ferredoxins. The *polymyxa* ferredoxin contains only one $Fe_4S_4^{2-}$ cluster, whereas the other ferredoxins contain two $Fe_4S_4^{2-}$ clusters. It would appear, therefore, that the two $Fe_4S_4^{2-}$ clusters in 8Fe-8S ferredoxins exhibit the same reactivity, or alternatively that the stability of the second cluster is dependent upon the integrity of the first. This phenomenon has been noted in the inability to reconstitute from apoprotein only one of the two $Fe_4S_4^{2-}$-cluster core ions in Clostridial type ferredoxins (Hong and Rabinowitz, 1967).

In discussing plausible mechanisms to account for the pH dependence of the lability of the $Fe_4S_4^{2-}$-cluster core ions of ferredoxins, it is convenient to consider the acid and base dependence separately. The simplest mechanistic scheme to account for acid hydrolysis is provided in eq 2.

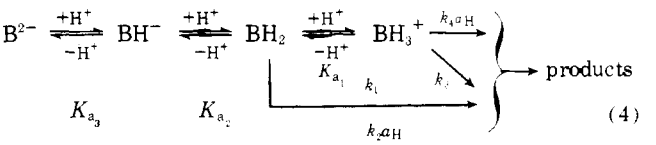


(2)

In eq 2, B^{2-} represents $Fe_4S_4(S\text{-}Cys)_4^{2-}$ etc., and the K_a's represent acid dissociation constants. Assuming equilibrium in B^{2-}, BH^-, BH_2, and BH_3^+, the scheme of eq 2 provides eq 3.

$$k_{obsd} = \frac{(k_3a_H^2 + k_2a_H + k_1K_{a_1})a_H^2}{a_H^3 + K_{a_1}a_H^2 + K_{a_2}K_{a_1}a_H + K_{a_3}K_{a_2}K_{a_1}} \quad (3)$$

In turn, eq 3 may be seen to be identical in mathematical form with the empirical equation employed to generate the log k_{obsd} vs. pH profiles for the ferredoxins below pH 6 (i.e., part I of eq 1). At the lowest pH values, the $k_3a_H^2/K_{a_1}$ term of eq 3 predominates and region A is second-order in hydrogen ion. Region B is provided by k_1, with the reaction again becoming second-order in hydrogen ion when $k_1a_H^2/K_{a_3}K_{a_2}$ predominates in region C. Unfortunately the reaction sequence of eq 2 is not unique. Kinetic equations of a mathematical form satisfactory to fit the data points of the pH-log k_{obsd} profile for the ferredoxins may also be obtained from the rather similar reaction schemes of eq 4 and 5, or from schemes which would



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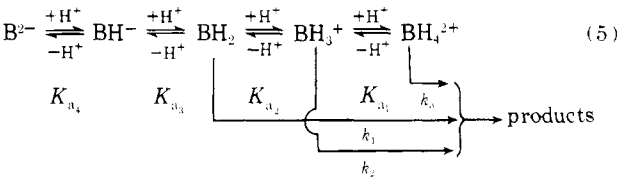

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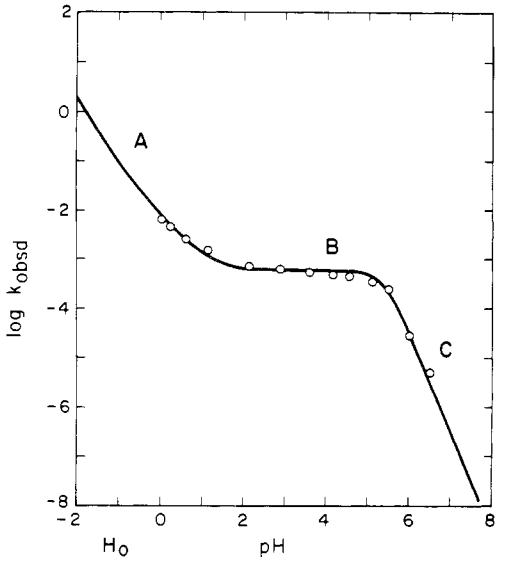

FIGURE 2: Log k_{obsd} vs. pH-rate profile for the dissolution of $Fe_4S_4(SCH_2CH(CH_3)_2)_4^{2-}$ in 60:40 vol % *N*-methylpyrrolidinone-water (values of k_{obsd} are in s^{-1}).

involve protonation at sites other than those of the clusters, e.g., pH-dependent conformational changes.

The usual dilemma that kinetics cannot differentiate between the site and the order of proton involvement in a reaction involving preequilibria is encountered here. Nevertheless, it can be stated with certainty that a series of preequilibrium protonations is required.

In a previous investigation of the hydrolysis of synthetic $Fe_4S_4(SR)_4^{2-}$ cluster ions (Bruice et al., 1975) (RS- = CH_3S-, $(CH_3)_2CHCH_2S$-, $(CH_3)_3CS$-), it was found that in the acid region the simplest fit of experimental points to the log k_{obsd} vs. pH profile was obtained by employing an equation derived from the kinetic sequence of eq 6 (ignoring all but acid-base equilibria).

$$B^{2-} \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} BH^- \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} BH_2 \begin{matrix} \xrightarrow{k_1} \\ \xrightarrow{k_2 a_H} \end{matrix} \Big\} \rightarrow \text{products} \qquad (6)$$

However, the pH dependence of the values of k_{obsd} for solvolysis of the models can be fit equally well by eq 3 which has been employed for the ferredoxins. Comparison of the pH profiles for dissolution of the $Fe_4S_4(S\text{-}Cys)_4^{2-}$ clusters of the ferredoxins (Figures 1A and 1B) and the model $Fe_4S_4(S\text{-}CH_2CH(CH_3)_2)_4^{2-}$ (Figure 2) establishes their identity or near identity. The inescapable conclusion is that the hydrolytic chemistry of the $Fe_4S_4(S\text{-}Cys)_4^{2-}$ moieties of ferredoxins corresponds to that of the model, and that chelation of an Fe_4S_4-cluster core ion to protein cysteinyl sulfurs does not change the mechanism of hydrolysis. Rate constants and acid-base equilibria are, however, influenced by the tertiary structure of the protein. Possible pH-dependent conformation changes apparently do not affect the observed rates since the same number of protons and same order of proton addition are required to decompose both protein bound and synthetic $Fe_4S_4^{2-}$-cluster core ions. That is, conformational changes need not be invoked to explain the observed kinetic behavior.

Constants employed to fit eq 3 to the experimental points for dissolution of the various proteins and the model compound $Fe_4S_4[S\text{-}CH_2CH(CH_3)_2]_4^{2-}$ at acid pH are presented in Table II. The rate constants k_1, k_2, and k_3 as well as pK_{a_1}, pK_{a_2}, and pK_{a_3} are surprisingly close for the $Fe_4S_4(S\text{-}Cys)_4^{2-}$ cores of *Cl. acidi-urici* and the model compound $Fe_4S_4[S\text{-}CH_2CH(CH_3)_2]_4^{2-}$. The average dielectric constant of the protein milieu surrounding the $Fe_4S_4(S\text{-}Cys)_4^{2-}$ moiety in *Cl. acidi-urici* may then be compared with 60:40 vol % *N*-methylpyrrolidinone-H_2O. Similarities in the rate constants of this Clostridial ferredoxin and the model $Fe_4S_4^{2-}$-cluster core ion suggest that the clusters of the ferredoxin are readily available to lyate species. The low reactivity of HIPIP can be explained by its $Fe_4S_4^{2-}$ cluster being shielded from solvent while the greater reactivities of the ferredoxins relative to the model compounds may be due to a less stable $Fe_4S_4^{2-}$ cluster brought about by the chelative geometry in the proteins. Differences in the structures of protein-bound Fe_4S_4 $(S\text{-}Cys)_4^{2-}$ clusters and the synthetic $Fe_4S_4(SR)_4^{2-}$ cluster ions have been realized by x-ray crystallographic studies (Averill et al., 1973).

TABLE II: Constants Employed to Fit Equation 3 to Experimental Rates of Ferredoxin Hydrolysis (Units in Seconds and Molarity).

Protein	pK_{a_3}	pK_{a_2}	pK_{a_1}	k_1	k_2	k_3
B. polymyxa	2.27	1.96	−0.30	40	10	20
C. vinosum	2.06	2.00	−1.00	1.3	10	25
Cl. acidi-urici [Leu][2]	3.30	3.00	−1.18	0.3	135	30
Cl. pasteurianum	3.48	2.70	−1.22	1×10^{-2}	16.7	25
Cl. acidi-urici	3.52	3.10	−0.92	8×10^{-3}	1.5	10
$Fe_4S_4(SCH_2C\text{-}H(CH_3)_2)_4$	3.92	5.82	−2.40	6×10^{-3}	20	0.5

TABLE III: Constants Employed to Fit Equation 11 to Experimental Rates of Ferredoxin Hydrolysis (Units in Seconds and Molarity).

Protein	k_1K_1	k_2	k_3	K_{HO}
B. polymyxa	1×10^{-5}	5×10^{-4}	20	120
Cl. pasteurianum	2.3×10^{-6}	7×10^{-4}	0.5	22
C. vinosum	1.5×10^{-6}	1×10^{-4}	1.3	20
Cl. acidi-urici [Leu][2]	1×10^{-6}	1.5×10^{-3}	0.6	15
Cl. acidi-urici	2.2×10^{-7}	8×10^{-4}	0.3	5
C. vinosum HIPIP	$<10^{-8}$	1×10^{-5}	1.0	0.015

No changes in initial absorbance of the characteristic chromophore of protein-bound $Fe_4S_4^{2-}$-cluster core ions at 390 nm were detected upon stopped-flow mixing (i.e., ~3 ms) of the various proteins with different acid buffers. The acid dissociation equilibria which are mandated by the pH behavior of the hydrolysis kinetics, e.g., K_{a_3} and K_{a_2} are therefore not spectrally detectable. The reported spectral detection of a pK_a for the $Fe_4S_4^{2-}$-cluster core ions of *C. vinosum* ferredoxin (Maskiewicz et al., 1975) was the result of an unfortunate operator provoked instrumental artifact.

The actual sites of protonation on $Fe_4S_4(SR)_4^{2-}$ ions were originally suggested to be at any one of the six faces of the $Fe_4S_4^{2-}$-cluster core ion as in I (Bruice et al., 1975). Protonation of the Fe_4S_4 moiety of $Fe_4S_4(S\text{-}Cys)_4^{2-}$ ions would be consistent with the greater charge density at core sulfurs relative to mercaptan sulfurs determined by MO calculations

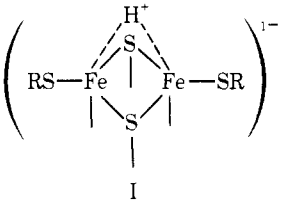

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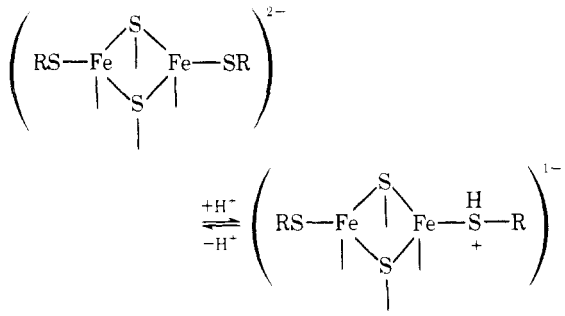
TABLE IV: Comparison of Reactivities of Protein Bound Fe_4S_4 Clusters with their Midpoint Redox Potentials (E_{mp}).

Protein	Rates[a] pH 2	Rates[a] pH 8	E_{mp}[b]	References
B. polymyxa	14.1	1.12×10^{-5}	−0.420	Stombaugh et al. (1973)
C. vinosum	0.56	2.16×10^{-6}	−0.489	Ke et al. (1974)
Cl. acidi-urici [Leu][2]	0.32	8.90×10^{-7}	−0.442	Lode et al. (1976b)
Cl. pasteurianum	0.016	1.75×10^{-6}	−0.390	Ke et al. (1974)
Cl. acidi-urici	0.010	2.19×10^{-7}	−0.442	Lode et al. (1976b)
C. vinosum HIPIP	2.51×10^{-6}		+0.350	Dus et al. (1967)

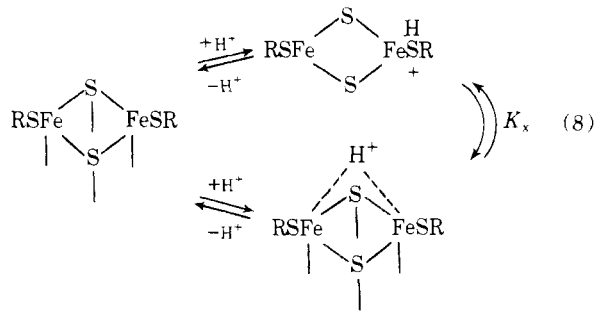
[a] Observed rate constants in s^{-1}. Maximum differences in rates between proteins were realized on the acidic plateau of the pH rate profiles, i.e., pH ~2. [b] Listed potentials were determined at pH 7–8.

(Yang et al., 1975). Such protonations would also be analogous to known examples of protonation of metals such as Ru^{2+} (Ford et al., 1968) and W^{0} (Olsen et al., 1974) in various complexes. However, x-ray studies upon *Peptococcus aerogenes* ferredoxin (Adman et al., 1975) and *C. vinosum* HIPIP (Carter et al., 1974b) have been interpreted to indicate the presence of hydrogen bonds between peptide amide hydrogens and the cysteine-sulfur ligands of the $Fe_4S_4^{2-}$ cluster. The constant pK_{a_3} may, therefore, pertain to the acid dissociation constant of RSH liganded to the $Fe_4S_4^{2-}$-cluster core ion (eq 7).



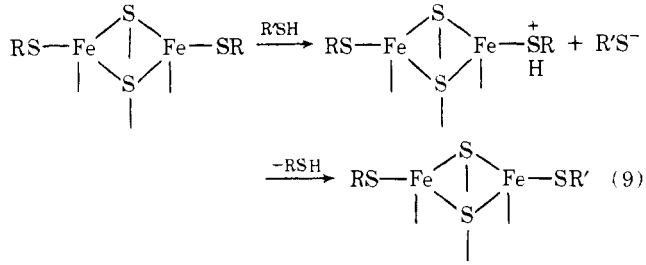
(7)

It is also possible that protonation takes place upon *both* the cluster core and the ligand (eq 8).



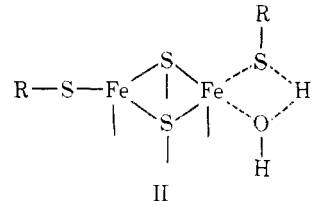
(8)

Thus, kinetic studies (acetonitrile solvent) of mercaptide ligand exchange have provided evidence that the exchange of -SR′ ligands with $Fe_4S_4(SR)_4^{2-}$ requires the general acid exchange of a proton between entering (HSR′) and leaving -SR moieties as in eq 9 (Dukes and Holm, 1975).



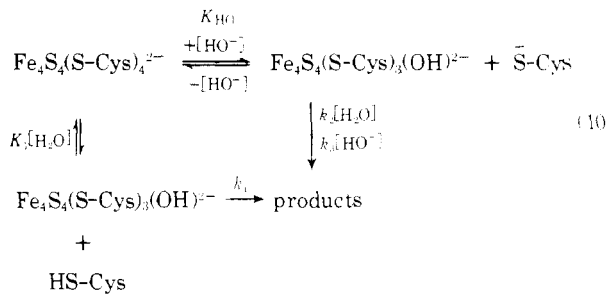
(9)

In aqueous solution, ligand exchange of -OH for -SR apparently arises via proton exchange between entering H_2O and departing -SR (Job and Bruice, 1975):



II

Below pH 5, the rates of dissolution of model $Fe_4S_4(SR)_4^{2-}$ complexes are not appreciably altered on addition of up to 200-fold excess of RSH over $Fe_4S_4(SR)_4^{2-}$. This result is best interpreted by assuming that the Fe_4S_4-cluster core ion itself is undergoing stepwise hydrolysis, and that each bond reorganization is preceded by protonation of the $Fe_4S_4^{2-}$-cluster core ion as in eq 2 (Bruice et al., 1975). On the other hand, model studies have shown that dissolution of $Fe_4S_4(SR)_4^{2-}$ above pH 6 is markedly inhibited by addition of RSH (Job and Bruice, 1975). Therefore, the reactions occurring at neutrality or at alkaline pH are best ascribed to ligand exchange. In this instance, the four RS- ligands are exchanged for HO^- (via II) prior to hydrolysis of the $Fe_4S_4^{2-}$-cluster core ion (Job and Bruice, 1975). At neutrality, extrusions of the $Fe_4S_4^{2-}$ clusters of partially denatured ferredoxins have been accomplished in the presence of excess mercaptan (Que et al., 1975). Possibly the simplest mechanism for dissolution of the $Fe_4S_4^{2-}$-cluster core ions of the ferredoxins at neutral to basic pH is provided by eq 10 (where K_1 and K_{HO} are association constants)



(10)

which provides the kinetic expression of eq 11a and 11b.

$$k_{obsd} = \frac{k_1K_1 + (k_2 + k_3[HO^-])K_{HO}[HO^-]}{K_1 + 1 + K_{HO}[HO^-]} \quad (11a)$$

$$k_{obsd} = \frac{k_1K_1 + k_2K_{HO}K_w/a_H + k_3K_{HO}K_w^2/a_H^2}{K_1 + 1 + K_{HO}K_w/a_H} \quad (11b)$$

Comparison of eq 11b with part II of eq 1 establishes their identity and the kinetic competence of the mechanism of eq 10. Possible pH-dependent conformational changes are not

required to explain the pH behavior of alkaline dissolution of protein-bound $Fe_4S_4^{2-}$-cluster core ions. Thus, the simplest mechanism consistent with the observed kinetics need invoke only ligand exchange equilibria which have been shown to occur during decomposition of synthetic clusters at basic pHs. The constants of eq 11, derived from the fit to the pH–log k_{obsd} profile at neutral and alkaline pH, are provided in Table III.

The dissolution of the $Fe_4S_4^{2-}$-cluster core ion of *C. vinosum* HIPIP is second-order in $[H_3O^+]$ at low pH and second-order in $[HO^-]$ at high pH. The unavailability of the cluster to solvent species provides great stability to HIPIP between pH 3 and 11 (Figure 1B). If one assumes that the mechanism for HIPIP dissolution is the same as for the ferredoxins (eq 10), then the equilibrium constant for HO^- addition (K_{HO}) to HIPIP is 3.3×10^2 to 8×10^3 times smaller than those found with the ferredoxins of this study. In the acid range, the linear pH–log k_{obsd} profile for HIPIP most likely is akin to region C of the profile for the ferredoxins. If so, the values of pK_{a_3} and pK_{a_2} would be required to be between 1 to 2 units lower than those of the ferredoxins (assuming $pK_{a_2} \cong pK_{a_3}$).

Differences in reactivity of $Fe_4S_4^{2-}$-cluster core ions in various iron–sulfur proteins, as kinetically determined in this study, should show a correlation with midpoint oxidation–reduction potential if the redox properties of the proteins are determined primarily by the same particulars which affect reactivity toward H_3O^+ and HO^-. However, no correlation of hydrolytic reactivity with E_{mp} (midpoint potential) is seen for the ferredoxins (Table IV). On the other hand, HIPIP does differ substantially from the ferredoxins in both midpoint redox potential and reactivity.

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