Reduction of Fe(III) Ions Complexed to Physiological Ligands by Lipoyl Dehydrogenase and Other Flavoenzymes in Vitro

IMPLICATIONS FOR AN ENZYMATIC REDUCTION OF Fe(III) IONS OF THE LABILE IRON POOL

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Enzymatic reduction of physiological Fe(III) complexes of the “labile iron pool” has not been studied so far. By use of spectrophotometric assays based on the oxidation of NAD(P)H and formation of Fe(II) (1,10-phenanthroline)_3Fe^3+ as well as by utilizing electron paramagnetic resonance spectrometry, it was demonstrated that the NAD(P)H-dependent flavoenzyme lipoyl dehydrogenase (diaphorase, EC 1.8.1.4) effectively catalyzes the one-electron reduction of Fe(III) complexes of citrate, ATP, and ADP at the expense of the co-enzymes NAD(P)H. Deactivated or inhibited lipoyl dehydrogenase did not reduce the Fe(III) complexes. Likewise, in the absence of NAD(P)H or in the presence of NAD(P)^+, Fe(III) reduction could not be detected. The fact that reduction also occurred in the absence of molecular oxygen as well as in the presence of superoxide dismutase proved that the Fe(III) reduction was directly linked to the enzymatic activity of lipoyl dehydrogenase and not mediated by O_2^- as previously reported. Kinetic studies revealed different affinities of lipoyl dehydrogenase for the reduction of the low molecular weight Fe(III) complexes in the relative order Fe(III)-citrate > Fe(III)-ATP > Fe(III)-ADP (half-maximal velocities at 348–485 μM). These Fe(III) complexes were enzymatically reduced also by other flavoenzymes, namely glutathione reductase (EC 1.6.4.2), cytochrome c reductase (EC 1.6.99.3), and cytochrome P450 reductase (EC 1.6.2.4) with somewhat lower efficacy. The present data suggest a (patho)physiological role for lipoyl dehydrogenase and other flavoenzymes in intracellular iron metabolism.

The biological importance of iron is substantially based on the fact that the function of numerous cellular proteins is coupled to the intramolecular presence of this transition metal. By far, the largest part of the intracellular iron is tightly bound to different proteins (e.g. ferritin or heme proteins), which are distributed over the variety of the intracellular compartments. However, a small part (0.2–3%) of the cellular iron constitutes “iron in transit,” which is complexed to low molecular weight organic chelators of relatively low affinities for iron ions, e.g. citric acid, amino acids, sugars, ascorbate, ADP, ATP, and other nucleotides or is loosely associated to proteins or membrane lipids (1–8). This fraction constitutes the metabolically and catalytically reactive iron of the cell and is commonly termed “labile iron pool” or “chelatable iron pool” (5, 6, 9, 10).

In contrast to the iron bound in iron storage or functional proteins, iron in transit has a substantial cytotoxic potential. This cytotoxicity is mainly related to the fact that it catalyzes the formation of hydroxyl radicals (‘OH) from hydrogen peroxide (H_2O_2, Reaction 1 (11, 12)) or forms high valent iron-oxygen (ferryl) species (13).

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{HO}^\cdot \quad \text{(Fenton reaction)}
\]

Reaction 1

Whereas H_2O_2 reacts only slowly with most biomolecules, the hydroxyl radical and the high valent iron-oxygen species, which may also be generated from molecular oxygen in competition with the classical Fenton reaction, react rapidly, often close to diffusion control, with virtually all biological molecules (12). Consequently, such reactive oxygen species-mediated, iron-dependent injurious processes are considered to be crucial pathogenetic factors for numerous diseases (5, 6, 10, 14, 15).

At least a small fraction of the iron in transit must exist within the ferrous (Fe(II)) oxidation state, which is the basis for the numerous metabolic functions of iron (e.g. in mitochondrial heme synthesis) but also for the participation in deleterious radical-generating reactions. In recent years evidence has accumulated indicating that both an increased concentration (14–22) as well as an increased re-reduction and thus redox-cycling of “reactive” iron ions (23, 24) can reinforce iron-mediated damaging of biomolecules and cells.

The intracellular maintenance of the Fe(II) oxidation state requires the presence of effective Fe(III)-reducing equivalents within the cell in order to counteract several (patho)physiological oxidizing processes, e.g. rapid reduction of molecular oxygen or H_2O_2 (5, 25). In recent studies ascorbic acid and glutathione, as well as NAD(P)H, α-keto acids, ferredoxin, O_2, and thioredoxin have been regarded as the intracellular reductants for Fe(III) (5, 6, 9, 23). More recently, however, doubt arose whether these “classical” reductants are really responsible for the intracellular reduction of the metabolically and catalytically reactive iron, at least under certain conditions. It has been proposed that as yet unidentified NAD(P)H-dependent redox enzymes can effectively reduce ferric ions to the ferrous state (23).

More than 1 decade ago, Shi and Dalal (27) demonstrated that lipoyl dehydrogenase, a NAD(P)H-dependent flavoenzyme that often is denominated as diaphorase (26), is able to catalyze the one electron reduction of Cr(IV) and V(V). Until now, how-
ever, the ability of this enzyme to reduce Fe(III) ions complexed to physiological ligands has not been studied, which is more surprising as lipoyl dehydrogenase has been reported to reduce the bioavailable standard one-electron oxidant [Fe(CN)6]3− very rapidly (26).

In the present work, we therefore utilized spectrophotometric techniques based on the oxidation of NAD(P)H and on the Fe(II)-dependent flavoenzymes as well as EPR spectroscopy to study whether lipoyl dehydrogenase (EC 1.8.1.4) from Clostridium kluyveri and from porcine heart is able to reduce Fe(III) ions of the labile iron pool. The ability of other NAD(P)H-dependent flavoenzymes (i.e., glutathione reductase, EC 1.6.4.2; cytochrome c reductase, EC 1.6.99.3; and cytochrome P450 reductase, EC 1.6.2.4) to reduce Fe(III) was studied for comparison. As both the reduction potential of Fe(III) and the kinetics of enzymatic iron reduction (may) largely depend on the (chelating) ligands bound to the Fe(III) center, comparative studies were performed with ferric iron ions coordinated to a variety of common intracellular low molecular weight iron ligands, namely citrate, ADP, and ATP.

**Experimental Procedures**

**Chemicals**—Citric acid trisodium salt (dihydrate) and l-ascorbic acid were obtained from Merck; ADP was from Fluka (Neu-Ulm, Germany); and catalase (6500 units mg−1 protein, from beef liver); ATP, NADH, NADPH, glutathione reductase (EC 1.6.4.2; 150 units mg−1 protein; from yeast), and aldehyde dehydrogenase (EC 2.1.2.1; 41.2 units mg−1 protein; from yeast) were from Roche Applied Science. The following reagents were from Sigma: Chelex 100 (chelating resin; iminodiacetic acid), 3,3′-methylene-bis(4-hydroxycoumarin) (dicumarol), 1,10-phenanthroline, FeCl3, Fe(NH4)2(SO4)2, ferric citrate, 1,5-methoxyindole-2-carboxylic acid ethyl ester (MICA), reduced GSH, superoxide dismutase, NADH, and lipoamide dehydrogenase (EC 1.8.1.4; synonyms: dihydrolipoamide dehydrogenase, diaphorase, lipoamide oxidoreductase (NADH), and lipoamide dehydrogenase) from C. kluyveri (diaphorase activity, 5.2 units mg−1 protein) and from porcine heart (Straub enzyme; diaphorase activity, 2.4 units mg−1 protein). Studies were performed with 0.05−0.5 units of lipoyl dehydrogenase ml−1 and 1 unit of “diaphorase” or “lipoyl” dehydrogenase (26) oxidizes 1 μmol of β-NADH min−1 (at pH 7.5, 25 °C) with the corresponding reduction of the electron acceptor 2,6-dichlorophenolindophenol as defined by the manufacturer. Concentrations of lipoyl dehydrogenase and of the other (flavoenzymes were calculated on the basis of their molecular masses as follows: lipoyl dehydrogenase, 100,000 Da (28); aldehyde dehydrogenase, 200,000 Da (29); cytochrome P450 reductase, 75,000 Da (30); glutathione reductase, 118,000 Da (31); cytochrome c reductase, 80,000 Da, (32). Comparative measurements were performed using identical enzyme side concentrations (= enzyme concentration × active sides).

All gas mixtures were delivered by Messer Griesheim (Oberhausen, Germany).

**Preparation of Fe(III) and Fe(II) Complexes**—Concentrated stock solutions of the Fe(III)-chelating ligands ATP, ADP, and citrate (1–100 mM each) were directly added from their stock solutions to the buffer molar excess of aqueous solutions of the ligands. In some experiments, the ligands were directly added from their stock solutions to the buffer solution (10−100 mM Tris buffer, pH 7.4), and then an aqueous solution of FeCl3 was added to give the above molar ratios. By this means precipitation of Fe(III) as insoluble Fe(III)-oxyhydroxide polymers at neutral pH was avoided. In some experiments a Fe(III)-citrate complex (500 μM) was transferred to a quartz cuvette containing Chelex 100-treated Tris buffer (10−100 mM, pH 7.4, 25 °C). Fe(III) oxidation was recorded by monitoring its UV/visible absorbance at 340 nm (UV/Visible Lambda 40, PerkinElmer Life Sciences) using Tris buffer as blank. After recording the Fe(III) base-line absorption for 5 min at 0.1-min intervals, lipoyl dehydrogenase from either C. kluyveri or porcine heart was added, and the absorption measurements were resumed. Afterward, the various Fe(III) complexes were added from their stock solutions to give the concentrations given under “Results.”

In experiments performed under hypoxic conditions, Tris buffer (6 ml containing NADH (5−200 μM) and either ATP, ADP, or citrate (5−200 μM) was transferred to a reaction tube that was continuously bubbled with argon by means of a cannula piercing the rubber stopper of the reaction tube. Afterward, Fe(III) (0.01–2.0 mM; as FeCl3) was added using a Hamilton syringe (see above). Before the addition of the reaction mixture was transferred with a Hamilton syringe to an argon-flushed quartz cuvette, and the recording of the absorption was started. After recording of the background absorption (E0), the sample, lipoyl dehydrogenase from either C. kluyveri or porcine heart (0.39 μM) or, for comparison, cytochrome P450 reductase, glutathione reductase, cytochrome c reductase, or aldehyde dehydrogenase, which is no flavoenzyme and served as control, was added to the reaction tube. NADH oxidation was recorded from this aliquot repetitively to the reaction tube at different time points. Immediately after a sample had been transferred to the cuvette, 2% SDS was added to the cuvette in order to terminate the enzymatic reaction. NADH oxidation was quantified from changes at E615 using a calibration rate. Curve. Rates of NADH oxidation (μM min−1) were evaluated from the initial (i.e. within the first 10 min) decrease of E615 after addition of the (flavoenzymes in order to ensure that enzymatic activity was not underestimated due to significant changes in substrate concentrations.

Further experiments with lipoyl dehydrogenase were performed in the presence of superoxide dismutase (SOD, 500 units ml−1) or catalase (500 units ml−1) or with NADPH instead of NADH. In some experiments in the absence of catalase, the enzymatic activity of lipoyl dehydrogenase was measured (30 min) of the enzyme with the inhibitors 3,3′-methylene-bis(4-hydroxycoumarin) (dicumarol, 200 μM (41, 42)), 1,5-methoxyindole-2-carboxylic acid ethyl ester (MICA, 200 μM (43, 44)), or 1,3-bis(chloroethyl)-1-nitrosourea (BCNU, 300 μM (45)).

**Determination of Fe(II) Formation Using Spectrophotometry**—Fe(II) formation was determined using an assay based on complexation with the strong iron chelator 1,10-phenanthroline (46). 1,10-Phenanthroline has a much higher binding affinity (apparent binding constant, Kapp) for Fe(II) (log Kapp = 21.0 (47)) than for Fe(III) (log Kapp = 14.1 (47)). In the presence of Fe(II) the chelator forms the red colored tris-ε-phenanthroline-Fe(II) ion (Fe(II)(1,10-phenanthroline)3+ with an absorption maximum at 510 nm, whereas the Fe(III)-1,10-phenanthroline complex hardly absorbs at this wavelength (46).

Because of the rapid autoxidation of Fe(II) in the presence of O2, Fe(II) formation was assessed in the absence of oxygen similarly as described above for the determination of lipoyl dehydrogenase-mediated
ated NADH oxidation. After incubation of the various Fe(III) complexes with either lipoyl dehydrogenase or cytochrome P450 reductase, glutathione reductase, cytochrome c reductase, or aldehyde dehydrogenase in the presence of NADPH, repetitively sampled aliquots from the reaction tube were transferred to a cuvette containing 2% SDS. Then 1,10-phenanthroline (1 mM; stock solutions 0.1 M in 100% ethanol) was added, and the absorption of the Fe(II) (1,10-phenanthroline) complexes was determined at 510 nm. The concentration of the Fe(II) formed was calculated from the absorption using a calibration curve obtained from 1,10-phenanthroline (1 mM) and Fe(II) standard solutions prepared from ferrous ammonium sulfate and ascorbate (see above). Rates of Fe(II) formation (μM min⁻¹) were determined from the initial increase of E₈₁₀ after addition of lipoyl dehydrogenase and the other (flav)enzymes, respectively. All values were corrected for background absorption, monitored in the absence of 1,10-phenanthroline.

Further experiments with lipoyl dehydrogenase were performed in the presence of SOD (500 units ml⁻¹) and catalase (500 units ml⁻¹) or with NAD⁺ or NADPH, respectively, instead of NADH. Quantification of Fe(II) using 1,10-phenanthroline was neither affected by the presence of the organic ligands (ATP, ADP, and citrate) nor by the presence of the flavoenzymes or SDS. Kinetic properties of Fe(III) reduction mediated by lipoyl dehydrogenase, cytochrome P450 reductase, glutathione reductase, or cytochrome c reductase, respectively, as well as enzymemediated NADH oxidation were calculated with GraphPad Prism (GraphPad Software, San Diego, CA).

**Determination of the Redox State of Iron Ions Using EPR Spectrometry**—Because (high spin) Fe(III) generally shows a characteristic, easily detectable EPR signal (48), EPR spectrometry is a widely used method to study Fe(III) reduction by recording the decrease in signal intensity to a broad and indistinct high spin or EPR-silent low spin Fe(II) signal (23, 49, 50). All experiments were performed in the strict absence of molecular oxygen in order to avoid the following: (i) one-electron reduction of O₂ to O²⁻ (which itself is a redundant for Fe(III)) by the flavin moiety of porcine heart lipoyl dehydrogenase (see below); (ii) rapid reoxidation of Fe(II) by molecular oxygen; and (iii) formation of insoluble Fe(III)-oxyhydroxide polymers at the concentrations required for EPR spectroscopy. In order to obtain Fe(III) EPR signals with a sufficiently low signal-to-noise ratio, relatively high concentrations of Fe(III) (50 μM) were employed.

EPR spectra were recorded at 77 K on a Bruker ESP-300E X-band spectrometer (Bruker, Rheinstetten, Germany) equipped with a TM₁₀ wide bore cavity. The instrument settings used were as follows: microwave frequency, 9.53 GHz; modulation, 10 G; signal gain, 2.5 × 10⁴; sweep range, 2000; sweep time, 6 min; modulation frequency, 100 kHz. Chelex 100-treated Tris buffer (5 ml, 100 mM, pH 7.4, 25 °C) was transferred to a reaction tube and continuously bubbled with argon to remove oxygen (see above). Then NADH was added (both 60 μM), or citrate (10 mM) was added from concentrated stock solutions followed by addition of 1 mM Fe(III) (as FeCl₃, dissolved in double-distilled water) using a Hamilton syringe. The solution was incubated until complex formation between Fe(III) and the ligands became visually evident from the disappearance of an initially formed white precipitate (in case of ATP and ADP) or from the appearance of a yellow color (in case of citrate), respectively. Then an aliquot (0.5 ml) of the reaction mixtures was quickly transferred to an argon-flushed Suprasil quartz tube (4 mm outer diameter) using a Hamilton syringe. The quartz tube was immediately immersed in a liquid nitrogen Dewar insert, and EPR measurements were started. After having recorded the base signal of the Fe(III) complexes, lipoyl dehydrogenase from either C. kluyveri (1.93 μM) or porcine heart (9.65 μM) was added from concentrated stock solutions to the reaction tube to start the enzymatic reaction. Aliquots (0.5 ml) were repetitively sampled from the reaction tube and frozen to 77 K at the time points given under “Results,” and the EPR signals of Fe(III) were recorded. In some experiments reduced glutathione (5 mM) or ascorbate (2 mM) instead of lipoyl dehydrogenase was used as reductant. Similar experiments were performed in the presence of SOD (500 units ml⁻¹) or catalase (500 units ml⁻¹). In another series of experiments, lipoyl dehydrogenase instead of NADH was added to the reaction mixture, and the reaction was started by addition of NADH. In further control experiments NADH was replaced by NAD⁺ or NADPH, respectively. Spectra were evaluated with the Bruker Win-EPR software and plotted using SigmaPlot 8.0 (SPSS Inc.).

**Results**—All experiments were repeated at least twice. Traces shown in the figures are representative of all the corresponding experiments. The data are expressed as means ± S.D.

**Fig. 1.** Effect of lipoyl dehydrogenase on NADH oxidation in the presence of low molecular weight Fe(III) complexes under normoxic conditions. NADH was added (from a concentrated stock solution) to a quartz cuvette containing Chelex 100-treated Tris buffer (10 mM, pH 7.4, 25 °C) to give a final concentration of 200 μM. NADH oxidation was determined under normoxic conditions by monitoring its absorbance (Absorb. at 340 nm in 0.1-min intervals. Arrow 1 indicates when lipoyl dehydrogenase (0.39 μM) from C. kluyveri was added. Addition of the Fe(III) complexes (60 μM) is indicated by arrow 2. Note that in the absence of Fe(III) NADH absorption is virtually unaffected by lipoyl dehydrogenase. Each trace shown is representative of two independent experiments.

**Effect of Lipoyl Dehydrogenase on NADH Oxidation in the Presence of Fe(III) Complexes**—In order to assess whether physiological Fe(III) complexes (Fe(III)-ATP, -ADP, and -citrate) may act as electron acceptors in an NADH-dependent lipoyl dehydrogenase reaction, we studied the effect of the complexes on NADH oxidation by spectrophotometric recordings of NADH oxidation at 340 nm under normoxic conditions. Addition of all Fe(III) complexes (60 μM) to Chelex 100-treated Tris buffer (25 °C, pH 7.4) containing 0.39 μM lipoyl dehydrogenase from C. kluyveri and NADH (200 μM) led to a rapid and linear decrease in NADH absorption (Fig. 1), indicating an enzymatic oxidation of NADH to NAD⁺ as a result of electron transfer to Fe(III). Lipoyl dehydrogenase from porcine heart, which has been reported to effectively use O₂ as an electron acceptor (51), strongly decreased NADH absorption already in the absence of Fe(III) (data not shown) and, therefore, was not further used in the spectrophotometric NADH assay. Iron(III)-induced NADH oxidation turned out to be markedly dependent on the nature of the added Fe(III) complex; the highest rate was obtained with Fe(III)-ATP and the lowest rate with Fe(III)-ADP. Addition of the ligands alone had no effect on the NADH absorption (data not shown), indicating that the electrons delivered by NADH were transferred to Fe(III). Interestingly, at normoxic conditions NADH was also completely oxidized when the concentration of the Fe(III) added was much lower (20 μM) than that of NADH (200 μM). This fact indicated a rapid redox cycling of the Fe(II) formed (see below). In line with this, almost no Fe(II) formation was detectable under normoxic conditions, i.e. 1.3 μM Fe(II) after 30 min in experiments with Fe(III)-ATP, whereas 120 μM NADH was oxidized within the same period (data not shown). Oxidation of NADH (μM min⁻¹) was linearly proportional to the concentration of the Fe(III) complexes up to at least 800 μM (in case of Fe(III)-ATP; data not shown).

The conclusion that the observed oxidation of NADH was related to the enzymatic activity of lipoyl dehydrogenase, i.e. originated from an enzymatic reaction, was confirmed by various control experiments. In the absence of the enzyme, addition of the Fe(III) complexes had almost no effect on NADH oxidation, indicating that NADH was not oxidized in a non-enzymatic fashion by direct electron transfer to Fe(III). This is
in line with the fact that NAD(P)H is a weak reductant for Fe(III) ions at neutral pH (23, 52). When the experiments were repeated with enzyme that had been deactivated by heating (100 °C, for 10 min), no decrease in NADH absorption was detectable (data not shown). The same behavior was observed, when lipoyl dehydrogenase was preincubated (for 30 min) with various inhibitors. Dicumarol (200 μM), a specific inhibitor of DT-diaphorase that binds to the oxidized form of the enzyme competitively versus reduced pyridine nucleotides (41, 42, 53), completely prevented NADH oxidation under the applied conditions (not shown). The same inhibitory effects were also found for the specific lipoyl dehydrogenase inhibitors MICA (200 μM), which appears to interfere with the enzyme-bound flavin (54), and BCNU (300 μM), a thiol-carbamoylating agent that releases 2-chloroethyl isocyanate, which in turn reacts with thiol groups located at the active site of the enzyme (45).

As lipoyl dehydrogenase has a much higher affinity for NADH than for NADPH (with NADPH as reductant, the reaction with lipoic acid is less than 5% that with NADH (51)), the requirement of NADH as the primary electron donor for the reactions observed was further studied with NADPH (200 μM) instead of NADH. As expected, NADPH was oxidized somewhat slower (~40%) than NADH but still remarkably fast when the Fe(III) complexes were added (data not shown).

Effect of Lipoyl Dehydrogenase on the EPR-detectable Signal of Low Molecular Weight Fe(III) Complexes—In order to confirm that the Fe(III) complexes of the physiological low molecular weight ligands were actually the acceptor of the electrons donated by NADH, EPR spectroscopic measurements were performed. The reported binding stoichiometries for ATP, ADP, and citrate to Fe(III) show a surprising variation, ranging from 1:2 (55) to 1:6 (56) for Fe(III)-ATP and from 1:1 to 1:4 for the Fe(III)-citrate complex, for example (57). Consequently, the binding stoichiometries and thus the chemical characteristics of the Fe(III) complexes under the present experimental conditions are undefined. Therefore, as under typical physiological conditions the ratio of the concentration of the free ligands and the “chelatable” iron ions is certainly high, an excess of the ligands over Fe(III) was employed in order to allow formation of “physiological” Fe(III) complexes.

As Fe(III)-ATP provided the strongest EPR signal, most experiments were performed with this complex. In the presence of ATP, Fe(III) showed the characteristic EPR spectrum of high spin Fe(III) (S = 5/2) with g-values of about 9.2 and 4.2, respectively (Fig. 2) (48). When either lipoyl dehydrogenase or NADH was added to the Fe(III)-ATP complexes, no changes of the EPR signals could be detected within 1 h, indicating that in the absence of oxygen neither NADH nor lipoyl dehydrogenase significantly reduce the complexed Fe(III) ions (data not shown). However, when both NADH and the enzyme (1.93 μM, from C. kluyveri) were present, the EPR signal intensity decreased rapidly (Fig. 2A). Because after addition of lipoyl dehydrogenase no other EPR signal could be detected within a

Tris buffer (5 ml, 100 mM, pH 7.4, 25 °C) with 10 mM NADH and bubbled with argon (for 15 min) to remove oxygen. Afterward, an aliquot (0.5 ml) of the solution was transferred to an argon-filled Suprasil quartz tube using a Hamilton syringe. Then the quartz tube was immediately immersed in liquid nitrogen (77 K), and EPR measurements were started. After having recorded the signal of the Fe(III) complex in the absence of lipoyl dehydrogenase, the reaction was started by adding lipoyl dehydrogenase (LipDH) either from C. kluyveri (1.93 μM; A) or from porcine heart (9.65 μM; C) to the reaction tube. Effects on Fe(III) signals were recorded from aliquots repetitively sampled from the reaction tubes at the time points indicated. B and D show the time-dependent decrease in EPR signal intensity as calculated from the displayed spectra. Spectra have been corrected for base-line drifts using SigmaPlot and are representative of at least two independent experiments.
range of the magnetic field of 500–4000 G, formation of low spin Fe(III) ($S = 1/2, g \sim 2$) appears unlikely. Also formation of EPR-silent Fe(III)-ATP-lipoyl dehydrogenase complex(es) can be ruled out because of the low concentration of lipoyl dehydrogenase compared with the concentration of Fe(III) ([lipoyl dehydrogenase]/[Fe(III)] = 1:500). Therefore, the most reasonable explanation for the decrease of the EPR signal is the reduction of Fe(III)-ATP to an EPR-silent, very likely low spin Fe(II) complex.

As expected for experiments in the absence of molecular oxygen, neither SOD nor catalase (both 500 units ml$^{-1}$) had any effect on the time dependence of the Fe(III)-ATP EPR signals, indicating that iron reduction was not mediated/influenced by $O_2$ and/or $H_2O_2$ (data not shown). As already observed in the spectrophotometric studies, the Fe(III)-ATP EPR signal decreased more slowly when NADH was exchanged by NADPH (data not shown), confirming the preference of NADH as a co-substrate for the enzymatic reduction pathway.

When lipoyl dehydrogenase from C. kluyveri was replaced by the enzyme isolated from porcine heart, essentially the same effect on the Fe(III)-ATP signal was observed (Fig. 2C). However, porcine heart lipoyl dehydrogenase led to a significantly slower decrease of the EPR signals, indicating a lower potential for Fe(III) reduction, in line with its lower diaphorase activity (see “Experimental Procedures”). Like the prokaryotic enzyme, mammalian lipoyl dehydrogenase also reduced Fe(III) more slowly when NADPH instead of NADH was added as an electron donor (data not shown).

In order to confirm that the above decrease of the Fe(III) EPR signals actually reflected iron reduction, the effects of the well known Fe(III) reductants GSH (5 mM) and ascorbate (2 mM) (12) on the Fe(III)-ATP spectrum were studied (Fig. 3, A–D). Somewhat surprising, addition of GSH barely decreased the EPR signal within the same time frame, thus appearing to be only a weak reductant for Fe(III) coordinated to ATP. In contrast, addition of ascorbate quite rapidly led to a decrease of the Fe(III)-ATP signal, in accord with the known high efficiency of ascorbate for Fe(III) reduction (12). In association with the observations made with lipoyl dehydrogenase, no other EPR signals could be detected after completion of the reaction, again pointing to the formation of low spin Fe(II).

In summary, both NAD(P)H oxidation, as followed by spectrophotometry, and Fe(III) reduction, as monitored by EPR spectrometry, strongly indicate that lipoyl dehydrogenase is capable of effectively reducing Fe(III) coordinated to low molecular weight ligands in an enzymatic reaction. However, due to the limitations of our EPR spectrometric approach to monitor faster time dependence (i.e. within the first 2–3 min after mixing of the reactants), quantitative and kinetic aspects of lipoyl dehydrogenase-mediated Fe(III) reduction were assessed in comparative studies using spectrophotometry and 1,10-phenanthroline as a probe for the Fe(II) formed.

Quantitative and Kinetic Properties of Lipoyl Dehydrogenase-mediated Reduction of Fe(III) Coordinated to Low Molecular Weight Ligands—Because of the lower ability of porcine heart

![Fig. 3. Effect of the non-enzymatic Fe(III) reductants glutathione and ascorbate on the EPR-detectable Fe(III)-ATP signal. Fe(III)-ATP (1 mM) was added to a reaction tube containing Chelex 100-treated Tris buffer (5 ml, 100 mM, pH 7.4, 25 °C) and bubbled with argon (for 15 min) to remove oxygen. Afterward, an aliquot (0.5 ml) of the solution was transferred to an argon-filled Suprasil quartz tube. The quartz tube was immediately immersed in liquid nitrogen and EPR measurements were started. After having recorded the base-line Fe(III)-ATP signal, either reduced glutathione (GSH, 5 mM; A) or ascorbate (AscH, 2 mM; C) were added to the reaction tube. Effects on the Fe(III) signal were recorded from aliquots repetitively sampled from the reaction tubes at the time points indicated. B and D show the time-dependent decrease in EPR signal intensity as calculated from the displayed spectra. Compare the similar changes of the Fe(III)-ATP signal in the presence of ascorbate and NADH-dependent lipoyl dehydrogenase, respectively. Spectra have been corrected for baseline drifts using SigmaPlot and are representative of at least two independent experiments; compare with Fig. 2, A and C.](http://www.jbc.org/)

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Flavoenzyme-mediated Fe(III) Reduction

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lipoyl dehydrogenase to reduce Fe(III) (see above), exclusively the enzyme from C. kluyveri was used to assess the properties of enzymatic Fe(III) reduction in the studies reported below.

After incubation of the various iron complexes (0.01–2.0 mM) with NADH (200 μM) and lipoyl dehydrogenase (0.39 μM) in Tris buffer (25 °C, pH 7.4) and in the absence of oxygen, the addition of 1,10-phenanthroline (1 mM), after prior termination of the enzymatic reaction with SDS (2%), led to a rapid increase of the absorption at 510 nm due to the formation of the red-colored \([\text{Fe(II)} (1,10\text{-phenanthroline})_3]^{2+}\) complex. Formation of Fe(II), as quantified using a 1,10-phenanthroline/Fe(II)-based calibration curve (Fig. 4, A and B), followed a linear dependence on incubation time (Fig. 4A). Reduction of Fe(III) was strongly dependent on the nature of the ligand and was accompanied by proportional oxidation of NADH (Fig. 4C). Notably, under hypoxic conditions the iron-induced oxidation of NADH by lipoyl dehydrogenase was diminished 2–4-fold compared with the experiments performed under normoxic conditions (compare Figs. 1 and 4C).

In line with the EPR results, in the absence of lipoyl dehydrogenase and/or NADH, almost no Fe(II) formation could be observed within the same time range (data not shown). At constant concentrations of lipoyl dehydrogenase (0.39 μM) and NADH (200 μM), simple Michaelis-Menten-like kinetics for the formation of Fe(II) (and consequently NADH oxidation) were found on variation of the concentrations of the respective Fe(III) complexes (Fig. 5, A and B). Variation of the concentration of NADH at constant concentration of Fe(III) (60 μM) demonstrated the high affinity of lipoyl dehydrogenase for NADH (200 μM) and oxidation of NADH (200 μM) from the enzyme oxidation (data not shown). The kinetic data revealed that the affinity of lipoyl dehydrogenase for Fe(III) is dependent on the nature of the ligand in the order Fe(III)-citrate > Fe(III)-ATP > Fe(III)-ADP, with the corresponding concentrations at the half-maximal velocity ranging from ~350 to 500 μM (Table I).

**Reduction of Physiological Fe(III) Complexes by Flavoenzymes Other than Lipoyl Dehydrogenase**—In order to determine whether other flavoenzymes are also capable of reducing physiological ferric chelates, we studied several abundant flavoenzymes in parallel with lipoyl dehydrogenase, namely NADPH-glutathione reductase (EC 1.6.4.2), which is another pyridine nucleotide-disulfide oxidoreductase that is phylogenetically closely related to lipoyl dehydrogenase and seems to share a common catalytic mechanism (58, 59)), NADH-cytochrome c reductase (EC 1.6.99.3, for which diaphorase action has been reported as well (32)), and NADPH-cytochrome P450 reductase (EC 1.6.2.4, which has been reported to be responsible for the reduction of several Fe(III) complexes by microsomes (60, 61)). The non-flavoenzyme aldehyde dehydrogenase (EC 1.2.1.5) was included as a control.

Glutathione reductase, cytochrome c reductase, and cytochrome P450 reductase were also catalyzing the one-electron reduction of Fe(III) complexes of citrate, ATP, and ADP at the expense of NAD(P)H (Table I), whereas, as expected, no Fe(III) was reduced by aldehyde dehydrogenase/ NADH (data not shown). Lipoyl dehydrogenase was found to be somewhat more efficient in Fe(III) reduction than the other flavoenzymes under comparable conditions.

**DISCUSSION**

In the present study we demonstrate that ferric iron ions of the labile iron pool coordinated to typical physiological low molecular weight ligands can be reduced through an \(\text{O}_2\)-independent enzymatic reaction that is mediated by lipoyl dehydrogenase and, with somewhat lower efficacy, also by other NAD(P)H-dependent flavoenzymes.

Lipoyl dehydrogenase is a ubiquitous NADH-dependent flavoenzyme that has been isolated from many species, where it is...
part of the mitochondrial pyruvate dehydrogenase and α-keto- 
glutarate dehydrogenase complexes (28, 62, 63). The two 
enzyme-bound flavins as well as the two substrate-reducible di-
sulfide groups have been clearly shown to represent the two 
prosthetic groups in the catalytic action of the enzyme (28). 

Free (not in a multienzyme complex) lipoyl dehydrogenase 
mediated Fe(III) reduction/NADH oxidation on the concentration of low molecular weight Fe(III) complexes and NADH. Fe(III)-ATP, Fe(III)-ADP, or Fe(III)-citrate (0.01–2.0 mM) were added to a reaction tube containing Chelex 100-treated Tris buffer (6 ml, 100 mM, pH 7.4, 25 °C) with 5–200 μM NADH and bubbled with argon (for 15 min) to remove oxygen. Afterward, an aliquot (1 ml) of the solution was transferred to an argon-filled quartz cuvette containing 2% SDS. A 1 mM solution of 1,10-phenanthroline was added 2 min later. Formation of Fe(II) and oxidation of NADH following addition of lipoyl dehydrogenase (0.39 μM) from C. kluyveri were determined from changes at $E_{510}$ and $E_{340}$, respectively, using 1,10-phenanthroline/Fe(II)- and NADH-based calibration curves (see legend to Fig. 4). A, the kinetic properties of Fe(II) formation in dependence on the concentration of Fe(III) ([NADH] = 200 μM) is shown; B shows the corresponding oxidation of NADH. C, the dependence of Fe(II) formation on the concentration of NADH ([Fe(III)-ATP] and [Fe(III)-ADP] = 60 μM) is given. Each trace shown is the average of two independent experiments; goodness of fits for all traces ($R^2$, with GraphPad Prism) is between 0.976 and 0.994.

Fig. 5. Dependence of lipoyl dehydrogenase-mediated Fe(III) reduction/NADH oxidation on the concentration of low molecular weight Fe(III) complexes and NADH.
found in mitochondria extract largely originates from the pyruvate dehydrogenase complex (64).

Lipoyl dehydrogenase plays a role in the oxidative decarboxylation of pyruvate and α-ketoglutarate, where the physiological function of the enzyme is to deoxidize enzyme-bound dihydrolipoic acid at the expense of NADH reduction (28). Besides its main activity, lipoyl dehydrogenase possesses a remarkably strong native diaphorase activity and therefore is often termed as diaphorase (26, 65, 66). The name diaphorase has been loosely attributed to several enzymes that catalyze the oxidation of either β-NADH or β-NADPH in the presence of an electron acceptor. A native diaphorase activity was found for lipoyl dehydrogenase from bovine intestinal mucosa and for the pig heart enzyme of Straub (26, 66).

In a recent study on extracellular microbial iron reductases, it has been suggested that the flavin group of flavoenzymes with diaphorase activity successively donates one electron and one proton to the substrate. This feature would explain the capability for reduction of metal ions (67). Because of the low redox potential of the FADH₂/FAD couple (−0.219 V, E° at pH 7 (68)) compared with the high redox potentials of most physiological Fe(III)/Fe(II) couples (69), reduced flavin adenine dinucleotide (FADH₂) should be one of the most effective biological reductants for Fe(III). This has been demonstrated recently by Woodmansee and Imlay (23) who found that in Escherichia coli an NADH-dependent flavin reductase is responsible for the rapid re-reduction of Fe(III) on H₂O₂ treatment. Differently from lipoyl dehydrogenase, however, porkaryotic flavin reductases reduce free flavin, and consequently, reduction of Fe(III) is mediated by free FADH₂ and not directly by the enzyme (69).

The mechanism of the lipoyl dehydrogenase-mediated reduction of physiological ferric chelates as reported here appears to be similar to the one described previously by Searls et al. (70). In diaphorase reactions, which generally are not mediated by the active center disulfides, NADH, the primary electron-donating cofactor of the enzyme, transfers a hydride anion to the enzyme-bound flavins, thereby yielding FADH₂ and NAD⁺ (70, 71). In a second step, FADH₂, which has been shown to accept and redonate only one electron (65), transfers an electron to the acceptor substrate (70), thereby producing FADH⁺ and low molecular weight Fe(II) complexes as reported here. Lipoyl dehydrogenase has been reported to be very selective for NADH (70–72). Therefore, the apparent high efficacy of NADPH as an electron donor in the reaction reported here may indicate that the rate-limiting factor is the slow oxidation of the reduced enzyme by iron. Therefore, a “ping-pong mechanism” may allow NADPH to be a sufficiently strong reductant. Given that GSH is a weak reductant of ATP-bound Fe(III) (see Fig. 3A), it appears very unlikely that the reduction of Fe(III) is significantly mediated by the thiol groups of the enzyme.

In the present study the lipoyl dehydrogenase-mediated formation of Fe(II) was demonstrated to be mainly modulated by the following: (i) the availability of molecular oxygen, (ii) the ligands bound to the ferric ion, and (iii) the electron-donating co-substrates, NADH or NADPH, and their concentrations. The fact that hypoxia strongly diminished the iron-induced oxidation of NADH by lipoyl dehydrogenase implies that under normoxic conditions electrons might be transferred to molecular oxygen by the enzyme flavins, thus yielding O₂⁻ (compare Figs. 1 and 4C). However, lipoyl dehydrogenase from C. kluyveri has been reported not to directly react with oxygen (61). This also became evident in our studies (see Fig. 1). Therefore, the increased oxidation of NADH under normoxic conditions may indicate either a rapid re-oxidation (i.e. redox-cycling) of enzyme-bound Fe(II) (most likely within the “enzyme-substrate complex”) or may indicate that Fe(II) serves as an electron bridge between the enzyme flavins and O₂, a kind of electron transport chain that has been described for iron/ascorbate and oxygen (25). Both processes would result in an enzyme-mediated production of O₂⁻ and, consequently, H₂O₂ provided O₂, Fe(III), and NAD(P)/H are present and could reinforce NAD(P)/H oxidation, e.g., via release of ‘OH radicals. The reduction of molecular oxygen to O₂⁻ has also been described in studies on lipoyl dehydrogenase-mediated one-electron reduction of other transition metal ions, namely Cr(VI) and V(V) (27).

In line with the increased rate of NADH oxidation in the presence of oxygen, under normoxic conditions formation of 1,10-phenanthroline-detectable Fe(II) was diminished by more than 90% as compared with experiments under hypoxic conditions (formation of 1.3 μM Fe(II) within 30 min under normoxic conditions and more than 13 μM Fe(II) under hypoxic conditions in experiments with Fe(III)-ATP). This low steady-state level of Fe(II) under normoxic conditions indicates that the rate of Fe(II) autoxidation in the presence of oxygen strongly exceeds the rate of enzymatic formation of Fe(II) when iron is bound to ATP, ADP, or citrate. The two major processes that are regarded to be responsible for the autoxidation of ferrous iron ions in a normoxic aqueous environment are the reduction of molecular oxygen to O₂⁻ (see above) and of H₂O₂ (to give ‘OH + OH⁻). Both reactions are known to be strongly depend-

### Table I

| Enzyme     | Fe(III) complex | Fe(III) concentration at half-maximal velocity | Kₘ (NADH or NADPH) | Vₘₐₓ (Fe) | Vₘₐₓ (NADH or NADPH) | Maximum turnover number (Fe) |
|------------|----------------|---------------------------------------------|-------------------|----------|----------------------|-----------------------------|
| LipDH      | Fe(III)-citrate | 346 ± 68                                    | ND*               | 2.8 ± 0.19 | 3.5 ± 0.09           | 0.12 ± 0.01                 |
| LipDH      | Fe(III)-ATP     | 403 ± 95                                    | 3.0 ± 0.46        | 3.8 ± 0.32 | 1.5 ± 0.06           | 0.16 ± 0.01                 |
| LipDH      | Fe(III)-ADP     | 485 ± 123                                   | 4.1 ± 0.78        | 2.7 ± 0.25 | 1.5 ± 0.06           | 0.12 ± 0.01                 |
| GSH-Red    | Fe(III)-citrate | 129 ± 24                                    | 47.1 ± 8.96       | 1.5 ± 0.07 | 0.57 ± 0.05          | 0.07 ± 0.01                 |
| GSH-Red    | Fe(III)-ATP     | 863 ± 263                                   | 58.2 ± 15.1       | 2.5 ± 0.32 | 0.71 ± 0.15          | 0.11 ± 0.01                 |
| GSH-Red    | Fe(III)-ADP     | 157 ± 36                                    | 40.7 ± 14.9       | 1.1 ± 0.07 | 0.38 ± 0.06          | 0.05 ± 0.01                 |
| Cytc-Red   | Fe(III)-citrate | 581 ± 214                                   | 21.7 ± 4.0        | 2.8 ± 0.41 | 2.2 ± 0.15           | 0.12 ± 0.02                 |
| Cytc-Red   | Fe(III)-ATP     | 649 ± 221                                   | 19.3 ± 4.8        | 3.6 ± 0.50 | 3.0 ± 0.25           | 0.15 ± 0.02                 |
| Cytc-Red   | Fe(III)-ADP     | 222 ± 22                                    | 25.8 ± 6.0        | 1.4 ± 0.04 | 2.8 ± 0.24           | 0.06 ± 0.01                 |
| CP450-Red  | Fe(III)-ATP     | 881 ± 262                                   | ND                | 0.9 ± 0.12 | ND                   | 0.04 ± 0.01                 |

* ND, not determined.
ent on the characteristics of the chelating ligand (12, 25, 40). The Fe(II) complexes of all of the low molecular weight ligands tested in this work have been reported to be redox-active, i.e. to reduce and thus consume O2 and H2O2 with different efficacies (25, 40, 55, 57, 73, 74). Therefore, kinetic properties of the lipoyl dehydrogenase-mediated Fe(III) reduction and NAD/H oxidation can be assessed only in the absence of oxygen (see below).

Looking at the enzymatic reaction in the absence of O2, our kinetic studies revealed different, albeit low, affinities of lipoyl dehydrogenase for the reduction of the low molecular weight Fe(III) complexes (Table I). This fact suggests that the suitability of Fe(III) to function as an electron-accepting substrate for lipoyl dehydrogenase is, as would have been expected, modulated by the chelating ligand. The differences in the kinetic properties obtained here are probably more the result of steric and electronic factors of the Fe(III) complexes rather than changes in the redox potentials. However, the variation of the Fe(III) (20 μM)/citrate ratio from 1:1 to 1:100 had no effect on the rate of Fe(III) reduction by lipoyl dehydrogenase, indicating that either only a 1:1 Fe(III)/citrate complex is formed under these experimental conditions or that changes in the binding stoichiometry for citrate to Fe(III) do not affect the electron transfer from lipoyl dehydrogenase to Fe(III) and, consequently, the accessibility of the bound iron.2 The maximum turnover numbers, as calculated from the Vmax values determined for the various Fe(III) complexes, revealed that lipoyl dehydrogenase reduces Fe(III) bound to ATP, ADP, or citrate with moderate velocity, about 2–4 orders of magnitude slower than DL-lipoic acid or DL-lipoamide are metabolized by the enzyme (26). However, in terms of iron reduction the calculated rates are remarkably high (see below). Most likely they are even underestimated because when comparing the formation of Fe(II) and the corresponding oxidation of NADH, about 2–3 mol of NADH are oxidized per mol of Fe(II) formed (compare Fig. 4, A and B). This apparent loss of electrons strongly indicates that traces of oxygen were still present in the reaction mixture after bubbling with argon, i.e. a fraction of the electrons might have been donated to O2. This conclusion was substantiated in experiments with the strong Fe(II) chelator 1,10-phenanthroline. This ligand binds ferrous ions (contrary to the physiological ligands studied here) in a redox-inactive form (40). Thus, when 1,10-phenanthroline was used as a ligand for Fe(III), a rapid lipoyl dehydrogenase-mediated formation of ferrous 1,10-phenanthroline could be observed, even in the presence of oxygen. Under hypoxic conditions, 2 mol of [Fe(II) (1,10-phenanthroline)]2–32+ were formed at the (explicable) expense of 1 mol of NADH.2

Besides lipoyl dehydrogenase, also glutathione reductase, cytochrome c reductase, and cytochrome P450 reductase proved to be capable of reducing physiological Fe(III) complexes in an enzymatic reaction. We propose that the most likely candidates (also) contributing to the reduction of intra- or extracellular ferric chelates belong to the family of pyridine nucleotide-disulfide oxidoreductases class I and II (e.g. glutathione reductase, trypanothione reductase, lipoamide dehydrogenase, mercuric reductase and thioredoxin reductase, alkyl hydroperoxide reductase, and cytochrome c reductase) and to the superfamily of flavoprotein pyridine nucleotide-cytochrome reductases (including ferredoxin:NAD+ reductases, plant and fungal NAD(P)H:nitrate reductases, NADH:cytochrome b5 reductases, NADPH:P450 reductases, NADPH:sulfide reductases, and a number of other flavoproteins). For most members of these enzyme families diaphorase activity and/or the involvement in cellular iron metabolism/reduction has already been shown in previous studies, albeit mostly indirectly. Detailed discussion of these studies is beyond the scope of this paper. Conclusively, our data strongly suggest that other enzymes than those belonging to the two classes of ferric reductases so far known (i.e. soluble procaryotic flavin reductases and membrane cytochrome b-like reductases of eukaryotes; for a review see Ref. 69) are significantly involved in the reduction of intracellular iron.

Ascorbate and glutathione are generally regarded to be responsible for the intracellular reduction of Fe(III) (12, 75, 76). Evaluation of our EPR and spectrophotometric data revealed that lipoyl dehydrogenase from C. kluyveri at a concentration of 1.95 μM reduces Fe(III) bound by ATP about 17.5-fold faster than 5 μM of GSH, whereas 2 μM ascorbate is about 10-fold more efficient in Fe(III) reduction than the enzyme (calculation based on half-lives; compare Fig. 2, A and C, with Fig. 3, A and C). For lipoyl dehydrogenase from porcine heart (9.65 μM) the enzyme out-competes GSH by a factor of 20, and ascorbate is 8 times more effective at the same concentrations. From these data one can estimate that lipoyl dehydrogenase at an apparent concentration of about 14 μM, as has been found in pig heart mitochondria (77), should reduce about 0.8 μM Fe(III)-ATP min–1, assuming a reasonable intramitochondrial concentration of 10–15 μM of chelatable iron (78). These calculations are based on the following: (i) by assuming simple Michaelis-Menten kinetics for the formation of Fe(II) (Fig. 5A); (ii) the fact that the formation of Fe(II) is linearly proportional to the enzyme concentration; and (iii) by assuming a water content of the tissue of 70% (79). Such a rate of intramitochondrial Fe(III) reduction by lipoyl dehydrogenase would be about 17–50-fold lower than the one estimated for the 200–600 μM ascorbic acid present in heart muscle (80). However, most cultured cells contain only very small amounts of ascorbate (81, 82), and the intramitochondrial concentration of ascorbate is usually lower than the cytosolic level. For example, the intracellular concentration of ascorbate in cultured L-929 fibroblasts was found to be ≤12 μM,3 whereas intracellular GSH concentrations range between 0.9 and 5.6 μM for various cell types (83), respectively. Therefore, besides ascorbate, lipoyl dehydrogenase may play a decisive role in the intracellular reduction of iron ions of the labile iron pool within the mitochondria of cultured cells. Because lipoyl dehydrogenase from C. kluyveri was significantly more efficient in Fe(III) reduction than pig heart lipoyl dehydrogenase, in prokaryotic cells reduction of Fe(III) by this enzyme may be even of higher relevance. Because of the activity of various other flavoenzymes the expectable significance of enzymes for the intracellular reduction of Fe(III) should be even higher. In line with this, the responsibility of classical reductants like GSH for intracellular reduction of the metabolically and catalytically reactive iron within the bacterium E. coli was questioned at least under certain conditions (23). The predominant biological relevance of lipoyl dehydrogenase for the intracellular enzymatic reduction of physiological ferric chelates should mainly arise from its intracellular concentration, which has been reported to be much higher as compared with the concentrations of the other flavoenzymes studied here (e.g. lipoyl dehydrogenase, 14 μM in pig heart mitochondria (77); glutathione reductase, 0.058 μM in human red blood cells to 1.6 μM in rat liver (79)).

With regard to the requirement of NAD(P)H for the flavoenzyme-mediated reduction of Fe(III), the very low Km values for these co-substrates suggest that within cells the

2 F. Petrat, S. Paluch, and H. de Groot, unpublished results.

3 C.-D. Badrakhan, F. Petrat, M. Kirsch, and H. de Groot, unpublished results.
enzymatic reduction of Fe(III) should be largely unaffected by (patho)physiological changes of the NAD(P)H levels (see Fig. 5C and Table I). Physiological intracellular NADH concentrations range from around 100 μM in rat liver endothelial cells to 4.0 μM in hepatocytes (84) and thus well exceed the concentrations required to achieve maximum Fe(III) reduction rates (Vmax) of the enzyme. However, since the concentrations of the Fe(III) complexes found to be high when the reaction proceeds with half-maximal velocity (see Fig. 5A and Table I), an increase of the intracellular concentration of metabolically active Fe(III) should decisively enhance the level/production of Fe(II), thereby initiating/enhancing radical-mediated damaging processes. The concentration of intracellular iron in transit is generally reported to be in the low µM range (1, 2, 6, 9, 14, 15). We found that in liver cells iron in transit is distributed over several subcellular compartments at concentrations ranging from 6 to 7 μM in the cytosol and from 9 to 12 μM in mitochondria (78, 85). In several studies an increase of the concentration of iron in transit has been made responsible for the iron-mediated damaging of cells (14–22). In this context we found a 2.5-fold increase (from 3.1 to 7.7 μM) of the pool of iron in transit during cold incubation of cultured cells (86), resulting in an enhanced damage of the cells. Such an increase of the concentration of redox-active iron would more than double the rate of flavoenzyme-mediated Fe(III) reduction, as estimated on the basis of Michaelis-Menten kinetics.

In summary, at present it appears that (i) NAD(P)H-dependent flavoenzymes are generally able to reduce physiological Fe(III) chelates in an enzymatic reaction at the expense of NAD(P)H oxidation, thus underlining the significance of intracellular enzymatic Fe(III) reduction, and that (ii) lipoyl dehydrogenase is likely to be a dominant catalyst of enzymatic iron reduction within the cell. The rate of this reduction process is modulated by the nature of the physiological iron-chelating ligands. As inside cells it is largely unknown to which physiological iron-chelating ligands the iron is bound, it may be that much better (or worse) physiological diaphorase substrates (i.e. Fe(III) complexes) do exist than those studied here. Knowledge of the mechanism of the intracellular reduction of Fe(III) by flavoenzymes should be of fundamental importance in view of iron metabolism and (patho)physiology.
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Reduction of Fe(III) Ions Complexed to Physiological Ligands by Lipoyl Dehydrogenase and Other Flavoenzymes in Vitro: IMPLICATIONS FOR AN ENZYMATIC REDUCTION OF Fe(III) IONS OF THE LABILE IRON POOL
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