Reactivity of Medium-Chain Acyl-CoA Dehydrogenase toward Molecular Oxygen†

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ABSTRACT: The free two-electron-reduced form of medium-chain acyl-CoA dehydrogenase is reoxidized by 120 μM molecular oxygen (50 mM phosphate buffer, pH 7.6, 2 °C) with a half-time of approximately 7 s. Reoxidation yields hydrogen peroxide as a major product with only traces of the superoxide anion. In contrast, enzyme reduced with octanoyl-CoA is extremely slowly reoxidized by oxygen, and so a series of 14 different substrate analogues have been tested to assess the structural factors responsible for this effect. Complexes with redox-inactive ligands such as 3-thia- and 2-azaoctanoyl-CoA lead to an approximately 3000-fold slowing of the rate of reoxidation of the free dihydroflavin form of the enzyme. Comparable ligands lacking the thioester carbonyl function are much less effective with rates some 1.3–4-fold slower than the free enzyme. The strong suppression of oxygen reactivity observed with certain ligands is probably not simply a steric effect but may reflect desolvation of the active site and consequent destabilization of the superoxide anion intermediate formed during reoxidation of the flavin. The profound differences in oxygen reactivity between acyl-CoA dehydrogenase and acyl-CoA oxidase and the unusual stability of certain flavoprotein semiquinones in air are discussed in terms of these thermodynamic and kinetic arguments.

Two distinct classes of FAD-linked enzymes, the mitochondrial acyl-CoA dehydrogenases and the peroxisomal acyl-CoA oxidases, convert acyl-CoA thioesters to their corresponding *trans*-2-enoyl derivatives during fatty acid oxidation. Both oxidase and dehydrogenase remove *pro-R* hydrogens at C-2 and C-3 of their thioester substrates with the reduction of the flavin prosthetic group (Biellmann & Hirth, 1970a,b; Kawaguchi et al., 1980):

$$[\text{E–FAD}_{ox}\cdot\text{acyl-CoA}] \rightleftharpoons [\text{E–FAD}_{2e}\cdot\text{enoyl-CoA}]$$

Both enzymes form charge-transfer complexes with a variety of acyl-CoA derivatives (e.g. 3-keto-, 3-thia-, and 3-oxa analogues; Coudron et al., 1983; Jiang & Thorpe, 1983; Wang & Thorpe, 1991), and both enzymes are susceptible to inhibition by analogues found to be mechanism-based inhibitors of the dehydrogenase (Coudron et al., 1983; Jiang & Thorpe, 1983; Powell & Thorpe, 1988).

Despite these close similarities, substrate-reduced dehydrogenase and oxidase exhibit profound (greater than about 10^6-fold) differences in their reactivity toward molecular oxygen (Jiang & Thorpe, 1983). The yeast acyl-CoA oxidase reacts with oxygen directly with a turnover number of about 2000 molecules of hydrogen peroxide formed per minute under standard assay conditions (Shimizu et al., 1979; Jiang & Thorpe, 1983). In contrast, the mitochondrial enzyme has evolved to suppress the intrinsic reactivity of free flavins toward dioxygen while funneling electrons safely to the respiratory chain through electron-transferring flavoprotein (ETF;[1] Thorpe, 1991). Rapid reoxidation of the dehydrogenase requires two molecules of ETF (Reinsch et al., 1980; Gorelick et al., 1985), and the interflavin electron transfer between redox partners has been shown to involve obligatory one-electron steps (Gorelick & Thorpe, 1986):

$$[\text{E–FAD}_{2e}\cdot\text{enoyl-CoA}] + 2[\text{ETF}_{ox}] \rightleftharpoons [\text{E–FAD}_{ox}\cdot\text{enoyl-CoA}] + 2[\text{ETF}_{1e}]$$

Enoyl-CoA product exerts a crucial role in the modulation of the reactivity of the reduced acyl-CoA dehydrogenase toward potential oxidants (Beinert, 1963; Gorelick et al., 1985; Lenn et al., 1990; Lehman & Thorpe, 1990). Product binding greatly slows the reoxidation of the reduced enzyme toward molecular oxygen (Beinert, 1963) but markedly accelerates one-electron transfers to ETF (Gorelick et al., 1985) and several nonphysiological one-electron oxidants of the enzyme (Lehman & Thorpe, 1990). How can product binding induce such divergent responses, particularly since the reoxidation of dihydroflavins by molecular oxygen is also believed to involve one-electron transfer reactions (Ballou et al., 1969; Kemal et al., 1977; Bruice, 1984; Massey et al. 1988)?

This paper examines the oxygen reactivity of the medium-chain acyl-CoA dehydrogenase from pig kidney in an attempt to explore some of these issues. In particular, we have extended the early work of Beinert and colleagues to assess what structural features of acyl-CoA ligands are most effective at suppressing oxygen reactivity of the reduced enzyme (Beinert & Page, 1957; Beinert, 1963). More generally, we hope that a comparison of acyl-CoA dehydrogenase and oxidase will help to elucidate those factors that are responsible for the modulation of the oxygen reactivity in flavoproteins (Massey & Hemmerich, 1980; Ghisla & Massey, 1986; Massey et al., 1988).

Materials and Methods

Materials. CoASH (lithium salt), acetyl-CoA, acetoacetyl-CoA, superoxide dismutase, glucose, glucose oxidase, 4-aminoantipyrine, catalase, horseradish peroxidase, and cytochrome *c* were from Sigma. Sodium dithionite was obtained from the Virginia Smelting Co., Portsmouth, VA. 5-Deazariboflavin and 5-deaza-FAD were generous gifts from Drs. Sandro Ghisla and Vincent Massey. The following acyl-CoA derivatives were prepared, purified by high-pressure liquid chromatography, and quantified as described previously: alkyl-SCoA thioethers (Ciardelli et al., 1981; Powell et al.,

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[1] Abbreviations: dH_{ox}, dH_{1e}, and dH_{2e}, oxidized, semiquinone, and two-electron-reduced forms of acyl-CoA dehydrogenase without regard to protonation state of the flavin prosthetic group; ETF, electron-transferring flavoprotein; P, trans-2-octenoyl-CoA product.

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1987); 3-thia- and 3-oxaoctanoyl-CoA and 3-thiaoctyl-SCoA (Lau et al., 1988); 2-octynoyl-CoA (Freund et al., 1985); 3-ketooctanoyl-CoA (Thorpe, 1986), and coenzyme A sulfonate (Nishimura et al., 1982; Powell et al., 1987). The synthesis of 2-azaoctanoyl-CoA, 2-azadithiooctanoyl-CoA and 2-oxabutyryl-CoA will be described in a subsequent publication (R. Wang, R. K. Brantley, and C. Thorpe, unpublished results). Medium-chain acyl-CoA dehydrogenase was purified from pig kidney as described earlier (Lau et al., 1986). 1,5-Dihydro-5-deaza-FAD-substituted enzyme was prepared and quantified as described previously (Thorpe & Massey, 1983; Ghisla et al., 1984). Native oxidized enzyme was treated with a 5-fold excess of the mechanism-based inhibitor 2-octynoyl-CoA and freed from excess reagent by ultrafiltration as in Powell and Thorpe (1988).

General Methods. Unless otherwise stated, all buffers were 50 mM potassium phosphate, pH 7.6, containing 0.3 mM EDTA. Static absorbance measurements were conducted by using Cary 219 and Hewlett-Packard 8452A spectrophotometers. Oxidized enzyme and its blue neutral semiquinone state were quantified by using extinction coefficients of 15.4 mM^{-1} cm^{-1} (446 nm) and 5.3 mM^{-1} cm^{-1} (570 nm), respectively (Thorpe et al., 1979; Lehman & Thorpe, 1990). Fluorescence titrations employed a Perkin-Elmer 650-10S instrument. Binding experiments were analyzed by using a nonlinear regression analysis program (F-curve), developed by Dr. J. Noggle of this department, or Enzfitter (Elsevier Biosoft).

Anaerobic Procedures. Solutions were deoxygenated by repeated flushing with nitrogen as described earlier (Gorelick et al., 1985). Enzyme was photoreduced with 3 μM 5-deazariboflavin in solutions of 50 mM phosphate buffer containing 5 mM EDTA, pH 7.6 (Massey & Hemmerich, 1978). In experiments involving the blue semiquinone form, care was taken to stop irradiation before the accumulation of significant levels of the fully reduced species (Thorpe et al., 1979).

Stopped-Flow-Spectrophotometry. The Kinetic Instruments stopped-flow instrument (2-cm path length absorbance cell) was prepared for anaerobic work as described earlier (Lehmann & Thorpe, 1990). Concentrations of oxygen in the aerobic tonometer were checked with a Yellow Springs Instrument Model 53 oxygen electrode. Air-saturated solutions were prepared at 20 °C and then cooled to 2 °C within the stopped-flow driving syringe. Data acquisition software was from OnLine Instruments Systems.

Quantitation of Superoxide and Hydrogen Peroxide. Superoxide anion formation was quantified by using an operational extinction coefficient of 18.4 mM^{-1} cm^{-1} for the reduction of cytochrome *c* at 550 nm in the stopped-flow instrument (Nishino et al., 1989). Acyl-CoA dehydrogenase (12 μM) was photoreduced and mixed with an equal volume of air-saturated buffer containing 13 μM cytochrome *c* and 40 μg/mL catalase. Control experiments were conducted with the same components under anaerobic conditions. Hydrogen peroxide concentrations were determined by the method of Allain et al. (1974), by adding 71 μM 4-aminoantipyrine, 71 μM phenol, and 14 μg/mL horseradish peroxidase to 12.4 μM dehydrogenase immediately after air oxidation at 25 °C.

Results

Figure 1 (panel A) shows the reoxidation of two-electron-reduced acyl-CoA dehydrogenase ($E\text{-}FAD_{2e}$; see Materials and Methods) after being mixed with an equal volume of air-saturated buffer (50 mM phosphate, pH 7.6, 2 °C). The overall spectral changes accompanying reoxidation are shown in Figure 1B. Reoxidation, monitored at 450 nm, is not particularly rapid under these conditions and is half-complete

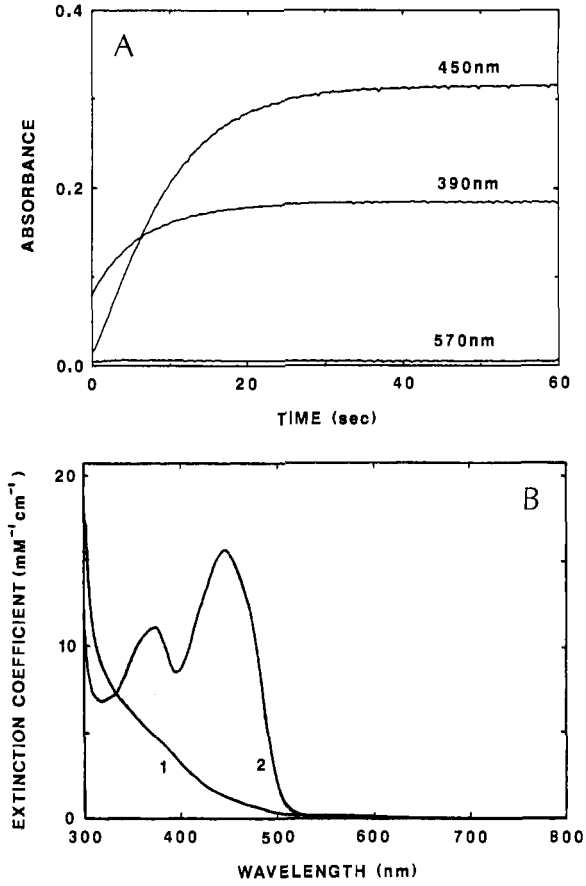


FIGURE 1: Reoxidation of the free dihydroflavin form of medium-chain acyl-CoA dehydrogenase by molecular oxygen. The reduced enzyme (20.4 μM enzyme flavin in 50 mM phosphate buffer, pH 7.6) was prepared by photoreduction (see Materials and Methods) and mixed with an equal volume of air-saturated buffer in a stopped-flow spectrophotometer at 2 °C. Reoxidation was followed at several wavelengths, of which 570, 450, and 390 nm are shown in panel A. Panel B compares the spectra of fully reduced and oxidized dehydrogenase (curves 1 and 2, respectively).

in about 7 s. A slight lag phase in the absorbance change is consistently observed at this wavelength with both photochemically reduced and dithionite-reduced enzyme (data not shown). Absorbance changes at 350 nm show a more pronounced lag, whereas 390-nm changes show little evidence of this feature (Figure 1A). The half-times for reoxidation of the enzyme are not strongly dependent on pH over the range 5.5–9.7 (with $t_{1/2}$ of 3–8 s; data not shown).

The kinetics of reoxidation (shown in Figure 1, panel A) are not significantly affected by the inclusion of 120 units/mL superoxide dismutase (see Materials and Methods; data not shown). Absorbance traces at 570 nm (Figure 1A) show that less than 2% of the total enzyme accumulates as the blue neutral semiquinone during reoxidation. This is in agreement with previous static titrations of the fully reduced dehydrogenase with air-saturated buffer (Thorpe et al., 1979; data not shown).

Experiments to trap superoxide released during reoxidation of the dehydrogenase by following the oxygen-dependent reduction of cytochrome c (see Materials and Methods) showed that about 3% of the maximal 2 moles of superoxide/dihydroflavin was formed. Essentially no direct reduction of the cytochrome occurred anaerobically (data not shown). Reoxidation of the photochemically reduced enzyme is accompanied by the formation of hydrogen peroxide as a major product (75% per enzyme flavin; Allain et al., 1974; see Materials and Methods) consistent with earlier work with enzyme reduced

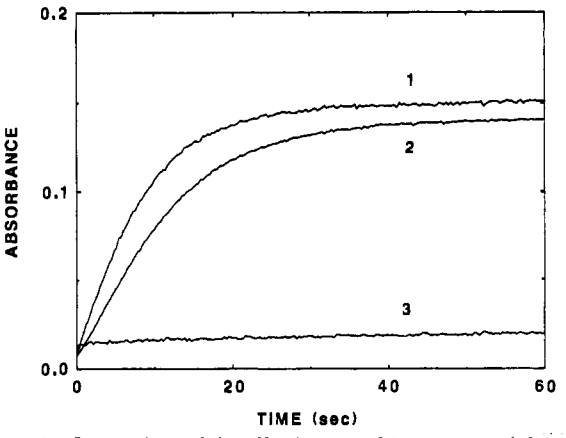
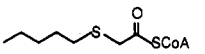
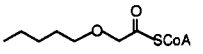
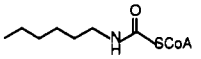
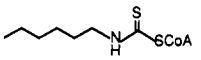
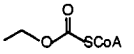
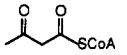
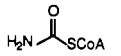
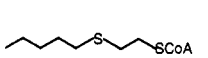
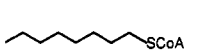
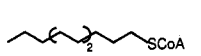
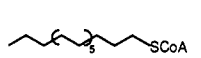


FIGURE 2: Comparison of the effectiveness of 2-azaoctanoyl-CoA and octyl-SCoA at protecting two-electron-reduced medium-chain acyl-CoA dehydrogenase from reaction with molecular oxygen. Enzyme (9.8 μM) was reduced and mixed with an equal volume of air-saturated buffer at 2 °C, as in Figure 1, containing no ligand (curve 1), 500 μM octyl-SCoA (curve 2), or 500 μM 2-azaoctanoyl-CoA (curve 3). Half-times for reoxidation at 450 nm are listed in Table I.

Table I: Effect of Acyl-CoA Analogues on the Reoxidation of Two-Electron-Reduced Medium-Chain Acyl-CoA Dehydrogenase by Molecular Oxygen[a]

No.	LIGAND	STRUCTURE	$t_{1/2}$ (sec)	$t_{1/2}$ ligand / $t_{1/2}$ free
	Ligand free	—	7	1
1.	3-thia-octanoyl-CoA		20000	2900
2	3-oxa-octanoyl-CoA		8100	1200
3.	2-aza-octanoyl-CoA		25000	3600
4.	2-aza-dithiaoctanoyl-CoA		28	4
5.	2-oxa-butyryl-CoA		1600	229
6.	acetoacetyl-CoA		300	43
7	2-aza-acetyl-CoA		370	53
8.	acetyl-CoA		450	64
9.	3-thia-octyl-CoA		90	12
10.	octyl-CoA		9	1.3
11.	decyl-CoA		19	2.7
12.	hexadecyl-CoA		60	8.5
13.	coenzyme A	HSCoA	7	1
14.	coenzyme A sulfonate	HO_3SCoA	7	1

[a] Reoxidation of the enzyme was monitored at pH 7.6, 2 °C, as in Figure 2 except for compounds 1, 2, 3, and 5, which were slow enough to be followed in a conventional spectrophotometer. Compounds 1–12 were used at 500 μM, whereas compounds 13 and 14 were present at 1 mM levels.

by β-(2-furyl)propionyl-CoA (McFarland et al., 1982).

The aim of the present work is not a detailed examination of the kinetics of this system but rather a characterization of the effects of various ligands on the overall reoxidation of the dehydrogenase. Hence we will compare ligands in terms of

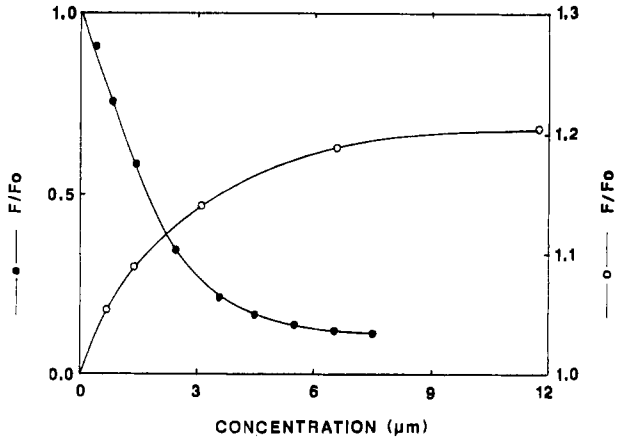


FIGURE 3: Fluorescence changes on the additon of acyl-CoA analogues to 1,5-dihydrodeaza-FAD-substituted acyl-CoA dehydrogenase. The reduced modified enzyme, in 50 mM phosphate buffer, pH 7.6, 25 °C, was prepared as described under Materials and Methods and titrated spectrofluorometrically with 3-thiaoctanoyl-CoA (closed symbols, left axis; 3 μM enzyme) and octyl-SCoA (open symbols, right axis; 1.4 μM enzyme). The ordinate shows the fluorescence expressed as a fraction of that observed before the addition of ligand. Curves correspond to dissociation constants of 1.45 and 0.12 μM for octyl-SCoA and 3-thiaoctanoyl-CoA, respectively.

Table II: Dissociation Constants for Binding Acyl-CoA Analogues to Native Oxidized Medium-Chain Acyl-CoA Dehydrogenase and Enzyme Substituted with 1,5-Dihydrodeaza-FAD

ligand	K_d (reduced)[a] (μM)	K_d (oxidized)[b] (μM)
3-thiaoctanoyl-CoA	0.12	0.47
2-azaoctanoyl-CoA	0.41	0.05
octyl-SCoA	1.45	4.0
decyl-SCoA	1.36	1.5

[a] The enzyme was reconstituted with 5-deaza-FAD and reduced with borohydride (Thorpe & Massey, 1983; Ghisla et al., 1984). Dissociation constants were determined from fluorescence titrations (see Results). [b] Dissociation constants were evaluated from spectrophotometric titrations of the oxidized native enzyme (see Results).

their effects on the time for half-completion of reoxidation at 450 nm.

Figure 2 shows the effect of 2-azaoctanoyl-CoA and octyl-SCoA (structures shown in Table I) on the reoxidation of the medium-chain acyl-CoA dehydrogenase monitored at 450 nm. Since reoxidation is very much slower than the binding of the ligands employed here (measured under the same conditions as Figure 2; Zhou and Thorpe, unpublished results), anaerobic solutions of photoreduced enzyme were routinely mixed with aerobic solutions of ligand delivered from a second tonometer (see Materials and Methods). Curve 3 shows that 500 μM of 2-azaoctanoyl-CoA markedly protects the dehydrogenase against reoxidation. This concentration appears to be saturating, since essentially the same protection is observed at a 10-fold lower level (data not shown). In contrast, the thioether, octyl-SCoA, offers little protection (curve 2) at 500 μM. Direct binding experiments (see below) suggest that it is also present at saturating levels.

An indication of the strength of ligand binding at the dihydroflavin level comes from titrations using 5-deaza-FAD-substituted enzyme. In contrast to the native reduced enzyme, the 5-deaza derivative is both fluorescent and air-stable in its reduced form, enabling binding to be monitored conveniently (Thorpe & Massey, 1983). Figure 3 shows that 3-thiaoctanoyl-CoA strongly quenches the fluorescence of the reduced flavoprotein whereas octyl-SCoA effects a minor enhancement. A similar divergent response to octanoyl-CoA and aceto-

acetyl-CoA was noted previously (Thorpe & Massey, 1983). Dissociation constants obtained from such data are summarized in Table II and compared with the corresponding values for the oxidized native enzyme. Three of the ligands bind somewhat more tightly to the reduced enzyme, whereas 2-azaoctanoyl-CoA is bound approximately 8-fold more weakly. Nevertheless, all of these ligands bind to the reduced enzyme with K_d values less than 1.5 μM. Hence the concentration of 500 μM employed in the bulk of these experiments is comfortably saturating. Thus the variation of protection observed in Figure 2 reflects differences of oxygen reactivity at the E–FAD_{2e}·L level and is not due to differences in the levels of residual free E–FAD_{2e} in these experiments.

The reoxidation experiments were extended to include the 14 ligands shown in Table I and either 0.5 or 1 mM concentration of acyl-CoA analogues. In each case, the ligand concentration used was considerably higher than the corresponding K_d for binding to the oxidized dehydrogenase, ranging from about 5-fold for the most weakly binding ligands (CoASH and acetyl-SCoA; Powell et al., 1987) to 10000-fold for 2-azaoctanoyl-CoA (Table II). In addition to freely dissociable ligands such as those shown in Table I, covalent modification of the enzyme with the mechanism-based inhibitor 2-octynoyl-CoA (Powell & Thorpe, 1988) exerts a profound slowing of the rate of reoxidation of the reduced enzyme by molecular oxygen (2600-fold under comparable conditions; see Materials and Methods). This result further supports the contention that the observed reactivities in Table I are largely those of complexed enzyme (see above).

A limited series of experiments with the semiquinone form of the medium-chain acyl-CoA dehydrogenase (see Materials and Methods; Thorpe et al., 1979) showed that the half-time for reoxidation by molecular oxygen under the conditions of Table I increases from 6 s for the free enzyme to more than 6 h in the presence of 500 μM 2-azaoctanoyl-CoA under the conditions of Table I. Obviously, protection of the same order of magnitude as that seen in Table I is also encountered at the semiquinone level.

Discussion

This paper extends the early observations of Beinert and Page (1957), who showed that propionyl-CoA, a nonsubstrate of the long-chain acyl-CoA dehydrogenase, markedly slows reoxidation of the two-electron-reduced enzyme by molecular oxygen (Beinert, 1963). The compounds listed in Table I exhibit a wide range of effectiveness in protecting the free reduced medium-chain enzyme (E–FAD_{2e}). These differences do not simply reflect the fraction of free reduced enzyme in these experiments (see Results) but correspond to the reactivity of each ligand bound species (E–FAD_{2e}·L) toward molecular oxygen.

The thioester carbonyl group is the single most important determinant in suppressing oxygen reactivity of the compounds tested in Table I. Thus compound 1 is some 200-fold more effective than the dithioether, compound 9, and about 2200-fold more effective than octyl-SCoA (compound 10). Replacing the carbonyl oxygen in compound 3 by a sulfur atom as in the dithioester compound 4 weakens the protection by about 900-fold. Indeed, the medium-chain acyl-CoA dehydrogenase accommodates dithioesters relatively poorly (C. Thorpe & V. Anderson, unpublished observations), in contrast to a number of acyl-CoA-requiring enzymes, where they serve as efficient substrates (Wlassics et al., 1988; Wrensford et al., 1991). The dissociation constants for compounds 3 and 4 to the oxidized native dehydrogenase (0.05 and 4 μM) further support the importance of the carbonyl group in the binding of these acyl-CoA analogues. Arguments will be presented later to suggest that the importance of the thioester carbonyl group is not simply a reflection of its capacity for restricting access to the flavin C-4a position.

In general, lengthening the alkyl chain increases the protection seen in Table I, although even acetyl-CoA (8) and its 2-aza analogue (7) offer significant protection (50–60-fold). Increasing the chain length from 2-azaacetyl-CoA to the 2-azaoctanoyl-CoA analogue (compound 3) improves protection by a further 70-fold. Even without the thioester carbonyl group, thioethers show a 6-fold increase moving from octyl- to hexadecyl-SCoA (compounds 10–12, Table I). In contrast, CoASH and $CoASO_3^-$ are ineffective up to 1 mM, although their K_d values for the oxidized enzyme (200 and 20 μM, respectively; Powell et al., 1987) would be expected to lead to significant complexation of the reduced enzyme.

In the succeeding sections we discuss some of the factors that might suppress the reactivity of flavoproteins toward molecular oxygen with particular reference to the acyl-CoA dehydrogenases. Reoxidation of two-electron-reduced flavins by oxygen is widely believed to be initiated by one-electron transfer with formation of superoxide and flavin radicals (Ballou et al., 1969; Kemal et al., 1977; Bruice, 1984; Massey et al., 1988). This process avoids the spin restriction imposed by a direct covalent reaction of dihydroflavin with molecular oxygen (Hamilton, 1971; Kemal et al., 1977), and is consistent with the extremely slow reoxidation of 1,5-dihydro-5-deazaflavins by molecular oxygen. Following one-electron transfer, the diradical pair can either dissociate with release of superoxide anion or collapse to a 4a-hydroperoxyflavin derivative with the eventual formation of oxidized flavin (Kemal et al., 1977; Bruice, 1984; Ghisla et al., 1977; Massey et al., 1988; Muller, 1983; Anderson, 1982). We note that recent pulse radiolysis experiments with glucose oxidase appear at variance with a simple recombination of superoxide and flavin radicals (Massey et al., 1988). Nevertheless, in the absence of a mechanistic alternative, we will consider the oxygen reactivity of the acyl-CoA dehydrogenase in terms of the widely accepted one-electron pathway.

Experimental and theoretical studies suggest that the interior of globular proteins is a dynamic matrix that can accommodate the rapid flipping of aromatic side chains and the facile diffusion of small neutral species such as oxygen or xenon (Lakowicz & Weber, 1973; Wuthrich & Wagner, 1979; Englander & Kallenbach, 1983; Tilton et al., 1984; McCammon & Harvey, 1987; Elber & Karplus, 1990). In particular, fluorescence quenching experiments suggest that no sector of a protein, or its prosthetic group, could be sequestered from multiple collisions with dioxygen over the time scale of minutes to hours required for the reoxidation of the acyl-CoA dehydrogenase (Lakowicz & Weber, 1973). Even assuming that transfer only occurs from sites on dihydroflavin with relatively high charge density (Dixon et al., 1979; Hall et al., 1987), it is hard to see how a ligand molecule occupying a fraction of one face of the isoalloxazine ring could protect all loci from attack. The problem is amplified if one admits the possibility that van der Waals contact between flavin and oxygen might not be necessary for electron transfer. Indeed, there is ample precedent for long-range electron transfers in biochemistry, particularly at highly favorable thermodynamic driving forces (Winkler et al., 1982; Sykes, 1985; Scott et al., 1985; Tollin et al., 1986; Gray & Malmstrom, 1989; Xia & Mathews, 1990; Mathews et al., 1991). Thus the rigorous exclusion of oxygen from loci within an enzyme·ligand complex appears to be improbable and therefore an unlikely mechanism for the

profound suppression of oxygen reactivity.

One factor likely to be a more important determinant in the reactivity of dihydroflavins toward oxygen is the solvation of the incipient superoxide anion radical. As would be expected for a reaction that converts an electroneutral species into a polar anion, the redox potential for the couple

$$O_2 + e^- \rightleftharpoons O_2^-$$

is strongly solvent dependent (Sawyer & Nanni, 1981): −330 mV in water (Hoare, 1985; −180 mV if 1 M oxygen in solution is used rather than the standard state of 1 atm of gaseous oxygen: Wood, 1988) compared to −500 mV in dimethyl sulfoxide (Sawyer & Roberts, 1966), −600 mV in pyridine (Sawyer & Nanni, 1981), and −640 mV in acetonitrile (Petersen & Evans, 1987). Reduction would be expected to be even less thermodynamically favorable in hydrocarbon solvents such as benzene or hexane (Reichardt, 1988; Sawyer & Nanni, 1981). Because of the anticipated relationship between the *rate* of electron transfer reactions and the thermodynamic driving force between reactants (Marcus & Sutin, 1985; Tollin et al., 1986), these differences would be expected to profoundly modulate flavin reactivity. Model studies showing large decreases in the reactivity of dihydroflavins toward molecular oxygen in apolar solvents are consistent with this view (Muller et al., 1975).

These arguments suggest that productive encounters with oxygen could be selected by manipulation of the local polarity within the active site of flavoproteins. Consider a dihydroflavin of redox potential comparable to the medium-chain acyl-CoA dehydrogenase (about −140 mV; Lenn et al., 1990; Gustafson et al., 1986) surrounded by a protein matrix that is unable to effectively solvate superoxide or to provide a proton to discharge its negative charge (Sawyer & Seo, 1977). Reduction of oxygen is likely to be highly unfavorable thermodynamically. Thus any superoxide formed is likely to return an electron to flavin faster than it could diffuse to the exterior of the protein. However, the provision of a more polar channel through which substrates would normally approach the N-5 region of the isoalloxazine ring would facilitate productive encounters with oxygen. Superoxide formation might then be focused at this locus and either recombine with the flavin radical to yield the hydroxyperoxy adduct or dissociate releasing superoxide anion in free solution.

Such solvent-accessible channels are liable to be desolvated to varying degrees upon binding substrate or suitable analogues. This decrease in polarity provides a mechanism for suppression of both superoxide anion formation and the subsequent steps in the reoxidation of flavin in the flavoprotein dehydrogenases. In the medium-chain acyl-CoA dehydrogenase, studies with a number of analogues suggest that the active site becomes significantly more hydrophobic upon ligand binding (Powell et al., 1987). The particular importance of the thioester carbonyl group revealed by the present study may reflect a conformational change induced on formation of a critical interaction between protein and ligand carbonyl oxygen. Alternatively, the carbonyl oxygen of effective ligands may occupy a locus that otherwise might stabilize the incipient superoxide anion. In contrast to the acyl-CoA dehydrogenase, the active site of the corresponding oxidase appears more polar, even in the presence of acyl-CoA ligands (Wand & Thorpe, 1991). In this case, product binding accelerates reoxidation of the flavin by molecular oxygen (R. Wang and C. Thorpe, unpublished results).

Because of the influence of thermodynamic driving force on reactivity in electron transfer reactions noted above, ligand-induced modulation of redox potential would be expected a priori to influence oxygen reactivity. The redox potential of E–FAD_{2e} is raised from −136 to −26 mV on binding *trans*-2-octenoyl-CoA (Lenn et al., 1990), making the one-electron reduction of oxygen much less favorable on thermodynamic grounds (see above). These thermodynamic and kinetic arguments may be releveant to a number of flavoproteins whose semiquinone state proves of exceptional stability toward molecular oxygen, e.g., yeast methanol oxidase (Mincey et al., 1980; Geissler & Hemmerich, 1981), electron-transferring flavoprotein from *Methylophilus methylotrophus* (Davidson et al., 1986), and lactate oxidase complexed with pyruvate (Choong & Massey, 1980). For example, the redox potential of the methylotrophic ETF shows an unusually postive value for transfer of the first electron (ETF_{ox}/ETF^-_{1e}) of +196 mV (Byron et al., 1989). Similarly, pyruvate binding to the anionic radical of lactate oxidase virtually eliminates oxygen reactivity (Choong & Massey; 1980) and, by preferential interaction with the semiquinone form of the oxidase (Choong & Massey, 1980), raises the one-electron redox potential from −67 mV (Stankovich & Fox, 1983) to about +74 mV. In contrast to these examples, flavoproteins relasing superoxide anion as a major product (Massey et al., 1969) would be expected to have suitably low 2e/1e potentials (Singer & Edmondson, 1974; Edmondson & Tollin, 1983) and to have evolved strategies to avoid the thermodynamically favorable collapse of the diradical pair (Ghisla & Massey, 1986; Massey et al., 1988).

Finally, it has been suggested that the purple-colored charge-transfer complexes formed between reduced flavin and enoyl-CoA product in the acyl-CoA dehydrogenases (Beinert, 1963; Lau & Thorpe, 1988) are important determinants in the suppression of flavin reactivity with oxygen (McFarland et al., 1982). Several observations argue against this proposal. First, a short-chain acyl-CoA dehydrogenase isolated from the anaerobic bacterium *Megasphaera elsdenii* forms strong charge-transfer complexes on the addition of butyryl-CoA and yet exhibits a relatively rapid oxidase reaction (Engel & Massey, 1971; Ellison et al., 1984). Second, 2-methyl-hexanoyl-CoA forms a sizable charge-transfer complex with the medium-chain acyl-CoA dehydrogenase and yet the resulting species is rapidly reoxidized in air (Cummings & Thorpe, unpublished results). Third, acyl-CoA oxidase forms significant charge-transfer complexes on the addition of preferred acyl-CoA substrates (Jiang & Thorpe, 1983) but reacts some million-fold faster with molecular oxygen than the dehydrogenase. Finally, none of the ligands employed in Table I yield charge-transfer complexes with the reduced dehydrogenase and yet many confer sizable protection against reoxidation. Thus charge transfer per se is not a sufficient condition for protection against oxygen reactivity.

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Role of the Four Conserved Histidine Residues in the Amidotransferase Domain of Carbamoyl Phosphate Synthetase†

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ABSTRACT: Carbamoyl phosphate synthetase from *Escherichia coli* catalyzes the formation of carbamoyl phosphate from ATP, bicarbonate, and glutamine. The amidotransferase activity of this enzyme is catalyzed by the smaller of the two subunits of the heterodimeric protein. The roles of four conserved histidine residues within this subunit were probed by site-directed mutagenesis to asparagine. The catalytic activities of the H272N and H341N mutants are not significantly different than that of the wild-type enzyme. The H353N mutant is unable to utilize glutamine as a nitrogen source in the synthetase reaction or the partial glutaminase reaction. However, binding to the glutamine active site is not impaired in the H353N enzyme since glutamine is found to activate the partial ATPase reaction by 40% with a K_d of 54 μM. The H312N mutant has a Michaelis constant for glutamine that is 2 orders of magnitude larger than the wild-type value, but the maximal rate of glutamine hydrolysis is unchanged. These results are consistent with His-353 functioning as a general acid/base catalyst for proton transfers while His-312 serves a critical role for the binding of glutamine to the active site.

Carbamoyl phosphate synthetase (CPS)[1] of *Escherichia coli* is a key enzyme in the biosynthetic pathways for arginine and pyrimidine nucleotides. This protein catalyzes the formation of carbamoyl phosphate according to the reaction

$$2\text{MgATP} + \text{HCO}_3^- + \text{H}_2\text{O} + \text{L-Gln} \rightarrow 2\text{MgADP} + \text{carbamoyl-P} + \text{P}_i + \text{L-Glu} \quad (1)$$

Although glutamine is the preferred nitrogen source, ammonia can be utilized as an alternate substrate at high concentrations.

The native enzyme is composed of two nonidentical subunits; a small one of molecular weight 41 270, and a large one of molecular weight 117 710 (Piette et al., 1984; Nyunoya & Lusty, 1983). The sole function of the small subunit is to hydrolyze glutamine for the production of NH_3, while the large subunit catalyzes the formation of carbamoyl phosphate from MgATP, HCO_3^-, and NH_3. The allosteric effectors (IMP, UMP, and ornithine) bind to the large subunit (Anderson & Meister, 1966) and control the activity of the enzyme by regulating the K_m of ATP.

The protein sequence of the heterodimeric enzyme of *E. coli* has been deduced from the nucleotide sequence of the *car*A (small subunit) and *car*B (large subunit) genes by Lusty and co-workers (Piette et al., 1984; Nyunoya & Lusty, 1983). Sequence comparisons of the small subunit have indicated that this protein belongs to the *trp*G-type glutamine amidotransferases (Piette et al., 1984; Nyunoya & Lusty, 1984; Werner et al., 1985). This class of enzymes also includes anthranilate synthetase (Nichols et al., 1980; Tso et al., 1980), *p*-aminobenzoate synthetase (Kaplan & Nichols, 1983; Kaplan et al., 1985), GMP synthetase (Zalkin et al., 1985), CTP synthetase (Wang et al., 1986), and formylglycinamide ribonucleotide synthetase (Schendel et al., 1989).

The *trp*G-type glutamine amidotransferases are all thought to involve an essential cysteine residue in the chemical mechanism for the hydrolysis of glutamine (Zalkin, 1985). The critical involvement of a cysteine residue in the amidotransferase activity of the *E. coli* CPS has been demonstrated by labeling experiments with a chloroketone analogue of glutamine (Khedouri et al., 1966; Pinkus & Meister, 1972) and cyanate (Anderson et al., 1973; Anderson & Carlson, 1975). All of the other *trp*G-type amidotransferase enzymes are inactivated by 6-diazo-5-oxo-L-norleucine (DON), an activated glutamine analogue (Levenberg et al., 1957; Patel et al., 1977), and it has been shown in most cases that a cysteine residue is labeled at the active site (Ohnoki et al.,

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[1] Abbreviations: CPS, carbamoyl phosphate synthetase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GMPS, GMP synthetase; AS II, anthranilate synthetase; PABS, *p*-aminobenzoate synthetase; CTPS, CTP synthetase.

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