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Regulation of the Redox Potential of General Acyl-CoA Dehydrogenase by Substrate Binding[†]

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Received September 28, 1989; Revised Manuscript Received December 11, 1989

ABSTRACT: Significant thermodynamic changes have been observed for general acyl-CoA dehydrogenase (GAD) upon substrate binding. Spectroelectrochemical studies of GAD and several of its substrates have revealed that these substrates are essentially isopotential for chain lengths of C-4 to C-16 ($E^{\circ\prime} = -0.038$ to -0.045 V vs SHE). When GAD is bound by these substrates, a dramatic shift in the midpoint potential of the enzyme is observed ($E^{\circ\prime} = -0.136$ V for ligand-free GAD and -0.026 V for acyl-CoA-bound GAD), thus allowing a thermodynamically favorable transfer of electrons from substrate to enzyme. This contrasts with values reported elsewhere. From these data an isopotential scheme of electron delivery into the electron-transport chain is proposed.

Acyl-CoA dehydrogenases catalyze the oxidation of acyl-CoA[1] esters to their *trans*-2-enoyl CoA products with concomitant reduction of the active site flavin in the first step of the fatty acid oxidation cycle. This cycle is an important source of ATP in biological systems, supplying up to 90% of the ATP in cardiac muscle (Bremer & Osmundsen, 1984). Thus, an understanding of the electron-transferring properties of this class of enzymes is imperative to understanding their role in regulating this vital energy production pathway. While a body of information has been amassed concerning the physiological importance (Tanaka, 1979) and physical (Auer & Frerman, 1980; Thorpe et al., 1979; Ikeda et al., 1985),

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[†] This work was supported by National Institutes of Health Grants GM29344 (M.T.S.) and GM40541 (H.L.).

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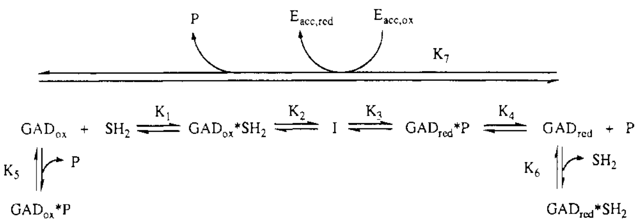
[1] Abbreviations: ACD, acyl-CoA dehydrogenases; BCD, butyryl-CoA dehydrogenase; CoA, coenzyme A; $\hat{E}$, conditional potential; E, measured potential; $E^{\circ\prime}$, midpoint potential; ETF, electron-transferring flavoprotein; FAD, flavin adenine dinucleotide; GAD, general acyl-CoA dehydrogenase; IDS, indigotin-5,5′-disulfonic acid; PYC, pyocyanin; FA-CoA, *trans*-β-2-furylacryloyl-CoA; FP-CoA, β-2-furylpropionyl-CoA; ITS, indigotrisulfonate.

0006-2960/90/0429-3709$02.50/0

Scheme I: Minimal Scheme for Acyl-CoA Dehydrogenase Turnover[a]



[a] GAD_{ox} = oxidized GAD, GAD_{red} = reduced GAD, SH_2 = acyl-CoA thioester substrate, P = *trans*-2-enoyl CoA product, $E_{acc,ox}$ = oxidized electron acceptor, and $E_{acc,red}$ = reduced electron acceptor.

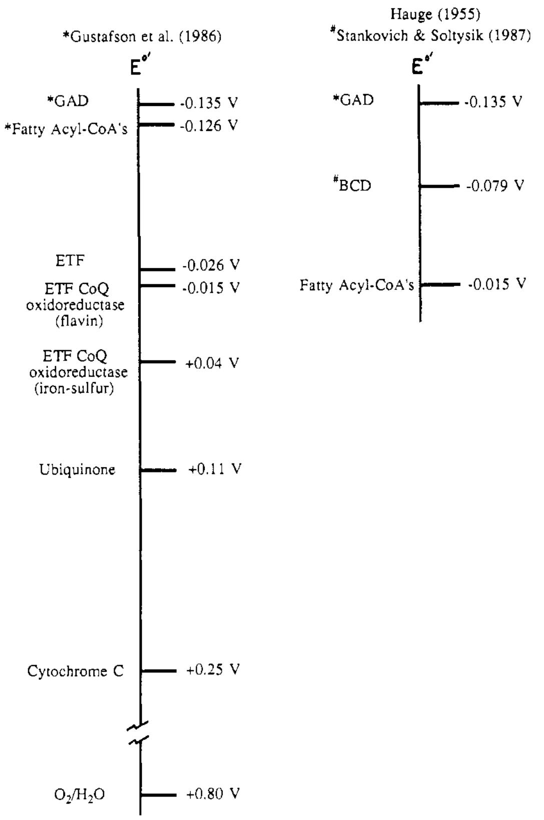
mechanistic (Lenn et al., 1989; Wenz et al., 1985), and structural properties (Kim & Wu, 1988) of this class of enzymes, some unresolved discrepancies concerning its electron-transferring properties remain.

A minimal scheme depicting electron transfer for general acyl-CoA dehydrogenase (GAD) is presented in Scheme I, as put forth by Schopfer et al. (1988). Binding of an acyl-CoA substrate to GAD results in a perturbation of the enzyme's visible spectrum. Following this, the substrate is oxidized, and a charge-transfer complex between the reduced flavin and product is formed. Reoxidation of the dehydrogenase flavin and product release are catalyzed by electron-transferring flavin protein (ETF). Electrons are then shuttled into the electron-transport chain via ETF-ubiquinone oxidoreductase, an iron–sulfur flavoprotein in this sequence. Each of the redox-active species shown in Scheme I has been spectrally characterized, making spectroelectrochemistry an ideal tool for probing this redox system. The spectral changes to GAD observed upon incubation with substrates, products, and analogues points to the significance of their binding in influencing this enzyme's electron-transferring properties (Thorpe et al., 1979; Powell & Thorpe, 1988; Lau et al., 1988).

Research into the electrochemical properties of this class of enzymes began in 1955 when Hauge reported the $E^{\circ\prime}$ of the product/substrate couple crotonyl-CoA/butyryl-CoA to be −0.019 V vs SHE (pH 7). Spectroelectrochemical investigation in our laboratory later confirmed this value and revealed that the enzyme butyryl-CoA dehydrogenase (BCD) exhibited an $E^{\circ\prime}$ = −0.079 V (pH 7). We were able to explain this seemingly unfavorable electron transfer from substrate to enzyme by probing the midpoint potential of a system containing the complexes BCD_{ox}–substrate/BCD_{red}–product in equilibrium. Our experiments revealed that upon binding of butyryl- and crotonyl-CoA to BCD, the $E^{\circ\prime}$ of BCD shifted positively to −0.018 V (pH 7), thereby allowing an energetically feasible pathway for substrate reduction to occur (Stankovich & Soltysik, 1987).

The evolutionary similarity between dehydrogenases from various bacterial and mammalian sources would suggest that straight-chain acyl-CoA dehydrogenases which differ only by their substrate chain length specificity would exhibit similar behavior. Additionally, it would be expected that the acyl-CoA substrates which vary only by carbon chain length would have similar $E^{\circ\prime}$ values. However, Gustafson et al. reported an $E^{\circ\prime}$ value of −0.126 V (pH 7.1) for the crotonyl-CoA/butyryl-CoA couple postulated from their work with a synthetic furanoyl-CoA and general acyl-CoA dehydrogenase (GAD). This value lies nearly isopotential with the $E^{\circ\prime}$ = −0.133 V (pH 7.1) for GAD (Gustafson et al., 1986), which is not bound by substrate or product.

Chart I: Two Theories of Electron Transport from Acyl-CoA Thioesters through Dehydrogenases into the Electron-Transport Chain As Represented by Reported Midpoint Potentials[a]



[a] Abbreviations: GAD, general acyl-CoA dehydrogenase; BCD, butyryl-CoA dehydrogenase; ETF, electron-transferring flavoprotein.

These contrasting results give rise to two views of electron transfer, which can be depicted in terms of midpoint values as shown in Chart I. In Chart I, the more negatively poised, and therefore better, reducing agents are at the top of each sequence; thus, the reducing equivalents should be passed from top to bottom in each case. The first possible pathway evolved from the work of Hauge (1955) in which the $E^{\circ\prime}$ values for the free enzyme (BCD and GAD) were found to be significantly more negative than those seen for the free substrates and products. To make the transfer of electrons from substrate to enzyme energetically feasible, a positive shift in the $E^{\circ\prime}$ of the enzyme must occur. Such a shift was observed for BCD upon binding of substrate and product (Stankovich & Soltysik, 1987).

The scheme put forward by Gustafson et al. (1986) represents an alternative pathway. Here the product/substrate couples are nearly isopotential with the unbound dehydrogenase, making reduction of the enzyme by the substrate readily accessible. However, there is then a larger free energy drop in the transfer of electrons from the reduced dehydrogenase to ETF ($E^{\circ\prime}$ = −0.025 V).

The discrepancy between these two theories has direct implications on the importance of enzyme binding in the regulation of electron transfer, the thermodynamics of dehydrogenase turnover, and the evolution of the various dehydrogenases' specificities. The following work will address these questions by investigating the redox potentials of product/substrate redox couples over a range of chain lengths and their influence on the redox properties of GAD.

Materials and Methods

Materials. General acyl-CoA dehydrogenase was purified from pig kidneys according to the method of Thorpe et al. (1979) with the addition of octyl-Sepharose column chromatography to aid in the removal of contaminating enoyl hydratase activity (Lau et al., 1986). Concentrations of holoenzyme and blue neutral semiquinone were measured spectrophotometrically with a molar absorptivity of 15.4 mM^{-1} cm^{-1} at 446 nm for oxidized GAD and of 4.1 mM^{-1} cm^{-1} at 560 nm for the blue neutral semiquinone (Thorpe et al., 1979). PYC was photochemically synthesized following the method of McIlwain (1937) from phenazine methosulfate purchased from Sigma. IDS was obtained from MCB Manufacturing Chemists at 89% purity, and methylviologen was purchased from British Drug House, Poole, England. Butyryl-, crotonyl-, octanoyl-, and palmitoyl-CoA were purchased through Sigma. *trans*-2-Octenoic acid was obtained from Aldrich and *trans*-2-hexadecenoic acid from ICN Biochemicals. Glass-distilled water from a Corning Megapure system was used in all experiments.

Preparation of CoA Thioesters. *trans*-2-Octenoyl-CoA and *trans*-2-hexadecenoyl-CoA were synthesized by the method of Bernet and Sprecher (1977). *trans*-2-Hexadecenoyl-CoA was purified by multiple aqueous and organic extractions on the basis of the procedure of Al-Arif and Blecher (1969). *trans*-2-Octenoyl-CoA was purified either by DEAE-Sephadex column chromatography (Williamson, 1983) or with a modification of the HPLC method of Eberhard (1987).

The HPLC system consisted of a Spectrophysics SP4290 integrator, SP8800 ternary pump, SP8450 UV/vis detector set at 260 nm, and a Rheodyne injector with a 100-μL sample loop. This system was interfaced to an Epson Equity 1+ microcomputer. Crude *trans*-2-octenoyl-CoA was lyophilized and then dissolved in a minimum volume of water, and the full loop capacity was injected onto a Vydac C-18 reverse-phase protein and peptide column. Pure octenoyl-CoA was eluted with a gradient of 15% methanol–85% 50 mM potassium phosphate buffer (pH 5.3) to 80% methanol–20% 50 mM phosphate buffer over 30 min. Following purification of octenoyl-CoA the column was washed with methanol to remove any residual impurities and then equilibrated with water. The purified CoA ester was then loaded directly onto the water-equilibrated column via the solvent inlet filter. After the column had been washed with water for 30 min to remove any phosphate salts, the octenoyl-CoA was then eluted by washing the column with 100% methanol. The collected *trans*-2-octenoyl-CoA showed a negative nitroprusside test and a 232:260 absorbance ratio of ~0.54, both of which are indicative of a pure CoA thioester (Stadtman, 1957a,b; Williamson, 1983). The pure CoA thioesters were stored lyophilized at −20 °C until needed at which time they were dissolved in water. These solutions were stored at −20 °C for several days.

Spectroelectrochemical Titrations. Anaerobic spectroelectrochemical cell methodology was as previously described (Stankovich, 1980; Stankovich & Fox, 1983) with either a Princeton Applied Research 173 potentiostat/179 coulometer or a Bioanalytical Systems 100 electrochemical analyzer and Apple 2+ or 2E interfaced Cary 210 or 219 spectrophotometers with thermostated cell compartments. All spectroelectrochemical experiments were done anaerobically at 25 °C in 50 mM potassium phosphate buffer (pH 7.6) containing 0.3 mM EDTA. PYC and IDS were electrochemically titrated in buffer and methylviologen (0.1 mM) in order to determine $E^{\circ\prime}$ and molar absorptivity under the described experimental conditions. An electrochemical titration of GAD (13.5 μM) was done to determine its $E^{\circ\prime}{}_{\mathrm{pH7.6}}$ with IDS (6.8 μM) and methylviologen (0.1 μM) as mediator dyes. All potentials are reported versus the standard hydrogen electrode (SHE).

Determination of Acyl-CoA Thioester $E^{\circ\prime}$. Three redox couples, crotonyl-CoA/butyryl-CoA, octenoyl-CoA/octanoyl-CoA, and hexadecenoyl-CoA/palmitoyl-CoA were examined independently to determine their midpoint potentials ($E^{\circ\prime}$). Experiments were performed as previously described (Stankovich & Soltysik, 1987), GAD being substituted for BCD and either the dye PYC (20 μM) or both PYC (20 μM) and IDS (5–10 μM) being used as redox indicators. IDS was substituted for ITS because its midpoint potential was nearer to both the GAD $E^{\circ\prime}$ measured in this laboratory and the value for the respective product/substrate redox couples reported by Gustafson et al. (1986). A known ratio of product/substrate for each redox couple (total concentration = 300 μM) was placed in the main cell compartment with the dyes and degassed. Following this, charge was added to reduce the PYC until the ratio of oxidized/reduced PYC was approximately the same as the ratio of product/substrate for the redox couple being investigated. This decreased the number of turnovers needed to reach equilibrium by poising the potential of the system near that of the final equilibrium and enhancing the rate at which equilibrium was attained. It did not, however, dictate what the final measured potential (E) of the equilibrating system would be, for even when the ratio of oxidized/reduced mediator dye was significantly different than that of the redox couple (prior to the enzyme addition) the E and final ratio of oxidized/reduced dye correlated well to the starting product/substrate ratio, thus indicating that the redox couple was indeed governing the system's equilibrium. Additionally, it had previously been shown that there is no electron transfer between the product/substrate mixture and the redox dyes in the absence of enzyme (Stankovich & Soltysik, 1987).

The equilibrium of the electrochemically reduced mediator dye mixture was monitored by the stability of the A_{690} (ΔA_{690} less than 0.0005/30 min) and the E. As this neared equilibrium, GAD (final concentration = 1–2 μM) was tipped in from the side arm and mixed thoroughly. Equilibrium of the system was considered to have been attained when the ΔE was less than 0.001 mV/30 min; this took less than 1.5 h.

HPLC Monitoring of CoA Thioester Redox Couples. The effect of enoyl hydratase activity on the equilibrium and ratio of product and substrate was monitored by HPLC. A means of separating the redox couples was developed on the basis of the methods of Corkey (1985) using 50 mM potassium phosphate buffer (pH 5.3) and methanol or acetonitrile for the mobile phase. Octenoyl-CoA/octanoyl-CoA were separated as described above. Crotonyl-CoA/butyryl-CoA required a gradient of 5–30% methanol over 30 min while a gradient of 60–75% acetonitrile over 15 min was used for hexadecenoyl-CoA/palmitoyl-CoA. Calibration curves for butyryl-, crotonyl-, and β-hydroxybutyryl-CoA (the product of crotonyl hydratase activity) were made in order to obtain accurate integrated peak areas for the described detector and column.

An aliquot of the redox couple mix was taken before and after GAD was added for comparison to the product/substrate ratio after equilibrium. Following the experiment an aliquot was removed from the cell compartment under positive nitrogen pressure and immediately analyzed by HPLC. The remaining experimental solution was treated with methanol (final concentration = 50%) and stored at −20 °C for possible later analysis. Equilibrium mixtures treated with methanol and stored gave identical product/substrate ratios to those analyzed immediately after the experiment for the crotonyl-CoA/butyryl-CoA and octenoyl-CoA/octanoyl-CoA couples.

Determination of $\hat{E}$ for Acyl-CoA Thioester Bound GAD. The midpoint potential of GAD when bound by either the crotonyl-CoA/butyryl-CoA, octenoyl-CoA/octanoyl-CoA, or hexadecenoyl-CoA/palmitoyl-CoA redox couple was determined as previously described for butyryl-CoA dehydrogenase (Stankovich & Soltysik, 1987). The effect of each redox couple was measured several times with a 1:1 ratio of product/substrate. Either PYC (20 μM) or both PYC (20 μM) and IDS (10 μM) were used as redox mediator dyes. Equilibrium was attained in less than 2 h for each couple. The ratio of product/substrate and effects of enoyl hydratase activity were monitored by HPLC as described above.

RESULTS

Potentiometric Measurements of GAD. The midpoint potential ($E^{\circ\prime}$) of GAD was measured by methods similar to those used by Gustafson et al. (1986), but with the described cell (Stankovich, 1980) in order to confirm that any discrepancy in reported potentials was not due to methodology differences or due to the fact that Gustafson's et al. results were obtained on the pig liver enzyme while we are working on the pig kidney enzyme. When GAD was titrated in the presence of IDS, it could be observed spectrally that the IDS ($E^{\circ\prime}_{\mathrm{pH7.6}}$ = −0.121 V) was fully reduced before the GAD had been completely titrated, which agrees with the $E^{\circ\prime}_{\mathrm{pH7.6}}$ = −0.136 V obtained for GAD. When this value is corrected to pH 7.1, the result is an $E^{\circ\prime}$ of −0.121 V. This $E^{\circ\prime}$ is within 0.017 V of that reported by Gustafson et al. (1986) for the pig liver enzyme. The two enzymes while alike are probably not identical, and this may be the cause of the 0.017 V difference in the potentials. Additionally, while the path of GAD reduction was similar to that reported by Gustafson et al., we observed slightly more blue neutral semiquinone (30% versus 20%). Similar comparisons can be made with the dyes PYC and IDS, which showed $E^{\circ\prime}$ values 0.013 and 0.021 V more positive than the values reported by Gustafson et al. (1986), respectively. Thus, the large variation in $E^{\circ\prime}$ values reported for the crotonyl-CoA/butyryl-CoA redox couple (>0.09 V) is not due to interlaboratory differences.

Potentiometric Measurements of Acyl-CoA Redox Couples. Experimental procedures developed previously for BCD (Stankovich & Soltysik, 1987) also applied well for GAD. Use of low enzyme concentrations (1 μM), relatively high redox couple concentration (300 μM), and a large ratio of mediator dye (10–20 × [GAD]) poised near the expected midpoint potential allowed rapid equilibration, thus reducing enoyl hydratase interference. A relatively large amount of mediator dye is necessary to keep the electrode poised in the presence of the large excess of the redox couple.

The combination of PYC ($E^{\circ\prime}_{\mathrm{pH7.6}}$ = −0.036 V) and IDS ($E^{\circ\prime}_{\mathrm{pH7.6}}$ = −0.121 V) in the cell compartment would encompass the range of $E^{\circ\prime}$ values reported by various laboratories for the crotonyl-CoA/butyryl-CoA redox couple (Hauge, 1955; Gustafson et al., 1986; Stankovich & Soltysik, 1987). If the $E^{\circ\prime}$ of any given redox couple was significantly more negative than that of PYC, then complete reduction of PYC and partial reduction of IDS would be expected upon equilibration due to the large excess of the redox couple to both of the dyes (>10:1). However, no reduction of IDS was observed with any of the redox couples examined. Rather, as seen in Figure 1, the measured potential values (E) were all within the range of PYC.

In Figure 1, a plot of the equilibrium potential versus the log [oxidized CoA thioester]/reduced CoA thioester] shows that all three redox couples exhibited Nernstian behavior for a total two-electron transfer, as evidenced by the slopes for each couple (ideal = 29.5 mV). The $E^{\circ\prime}_{\mathrm{pH7.6}}$ values and slopes (shown in parentheses) observed for each redox couple in Figure 1 are crotonyl-CoA/butyryl-CoA = −0.045 V (30 mV), octenoyl-CoA/octanoyl-CoA = −0.041 V (29 mV), and hexadecenoyl-CoA/palmitoyl-CoA = −0.038 V (28 mV).

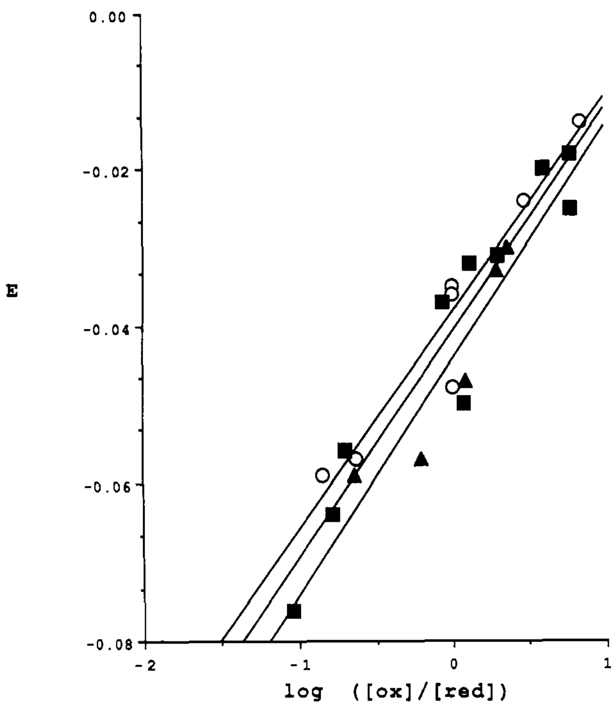


FIGURE 1: Nernst plots of acyl-CoA redox couples: (▲) crotonyl-CoA/butyryl-CoA; (■) octenoyl-CoA/octanoyl-CoA; (○) hexadecenoyl-CoA/palmitoyl-CoA.

Equilibration of the redox couple systems occurred within 1.5 h of addition of GAD and was stable for a minimum of 2 h. Disturbing this equilibrium by the addition of either oxidative or reductive charge resulted in reequilibration back to within 0.002 V of the original equilibrium potential. This indicated that the redox couple was serving to buffer the system potential and that the dye was communicating this potential. Enoyl hydratase activity, when present, resulted in consumption of the *trans*-2-enoyl CoA product. This was most noticeable after equilibrium had been reached and was detected (1) by a slow negative potential shift (0.001–0.002 V/h), caused by reoxidation, and (2) through HPLC analysis.

While octyl-Sepharose column chromatography removed a majority of the enoyl hydratase activity toward octenoyl-CoA, a small intrinsic enoyl hydratase activity is maintained toward crotonyl-CoA (Lau et al., 1986). Allowing the experimental mixture to set for a long period beyond that needed to reach equilibrium resulted in a steady conversion of crotonyl-CoA to β-hydroxybutyryl-CoA. This was quantified by HPLC, and in the few cases where the amount of β-hydroxybutyryl-CoA formed was significant, the ratio of crotonyl-CoA/butyryl-CoA at equilibrium could be approximated by adjusting for the β-hydroxybutyryl-CoA formed. No hydratase activity was seen toward hexadecenoyl-CoA; however, the *trans*-2-hexadecenoyl solution was not stable over long-term storage as evidenced by HPLC analysis. Nonetheless, even without HPLC confirmation on all samples, using the ratio of product/substrate measured prior to the experiment gave reasonable and reproducible results.

Potentiometric Measurements of Product/Substrate-Bound GAD. As mentioned previously, work in this laboratory with BCD from the bacteria *Megasphaera elsdenii* had shown that the binding of the crotonyl-CoA/butyryl-CoA redox couple to BCD caused the enzyme's $E^{\circ\prime}$ to shift positively with respect to free BCD. To test if GAD would exhibit similar behavior, identical experiments were performed with both PYC and IDS as mediator dyes. The $E^{\circ\prime}$ of these two dyes brackets a range of potentials expected for either a modified or unmodified $E^{\circ\prime}$ of GAD. In these experiments PYC was half-reduced in the presence of GAD, and a 1:1 mixture of the redox couple being examined was then added from the side arm. Addition of the redox couple of interest to the GAD, PYC, and IDS mixture resulted in both the rapid reduction of GAD and formation of a charge-transfer complex between GAD and product as indicated by the appearance of a long-wavelength band at 570 nm. The amount of charge-transfer complex varied for each of the different redox couples examined. Equilibrium was reached within 1.5 h for all of the substrates. Addition of oxygen to the system resulted in rapid reoxidation (10–15 min) of a majority of the GAD spectrum. This was followed by a slow reoxidation of the remaining spectrum over the next 1–2 h. The absorption maximum of GAD shifted red both during equilibrium and after reoxidation, which is indicative of the binding of product to oxidized GAD.

As shown in Figure 2 the extent of flavin chromophore bleaching and the amount of charge-transfer species formed were different for each of the three redox couples with the optimum substrate, octenoyl-CoA/octanoyl-CoA, effecting the largest percentage of reduction [% reduction = $(1 - A_e/A_i) \times 100$; A_i = initial absorbance at 446 nm; A_e = absorbance at 446 nm at equilibrium]. The same bleaching pattern was observed previously during anaerobic titrations of the enzyme with each of the three different substrates (Thorpe et al., 1979) and reflects where the equilibrium of K_2 for each substrate lies in Scheme I. During the titrations of GAD by various substrates (Thorpe et al., 1979) the optimal substrate favors the formation of $GAD_{red}{\cdot}P$. However, unlike those simple substrate titrations, a substantial amount of product-bound GAD_{ox} was present in the experimental conditions described herein due to the equimolar ratio of substrate and product initially mixed with oxidized GAD.

When the octenoyl-CoA/octanoyl-CoA redox couple was mixed anaerobically with GAD, an $E = -0.052$ V was observed at equilibrium, and the spectrum was bleached 71%. These data can be evaluated with the Nernst equation for $\hat{E}$, where $\hat{E}$ is the conditional potential. The conditional potential refers to the midpoint potential of the enzyme under a given set of conditions. In the experiments described in Figure 2, any of the enzyme species shown in Scheme I could be in the equilibrium mixture of interest; however, under the conditions used this system should contain $GAD_{ox}{\cdot}P$, $GAD_{ox}{\cdot}S$, and $GAD_{red}{\cdot}P$ primarily. Molar absorptivities for these species were extracted from the stopped-flow work of Gorelick et al. (1985) and Schopfer et al. (1988) and are $\epsilon_{450} = 14.1$ mM^{-1}, $\epsilon_{450} = 14.1$ mM^{-1}, and $\epsilon_{560} = 2.95$ mM^{-1}, respectively. By use of these values an $\hat{E}$ of −0.026 V was obtained.

The hexadecenoyl-CoA/palmitoyl-CoA redox couple gave considerably less bleaching of the flavin spectrum (6% and 26% in two different experiments) than either of the other two redox couples with measured potentials of −0.032 and −0.058 V, respectively. Furthermore, little or no charge-transfer species was observed. An $\hat{E} = -0.067$ V was obtained for both experiments when the same molar absorptivity as for octenoyl-CoA/octanoyl-CoA was used. The lack of flavin bleaching by this redox couple can be explained in part by micelle formation. Since the critical concentration of palmitoyl-CoA needed to form a micelle is below 12 μM (Thorpe et al., 1979), a significant amount of micelle formation would be expected under the experimental conditions used. It is known that GAD is inhibited by micelles. Their presence is confirmed by the red shift in the flavin spectrum and the enhanced resolution of the 450-nm band, both of which are indicative of increased hydrophobicity at the active site.

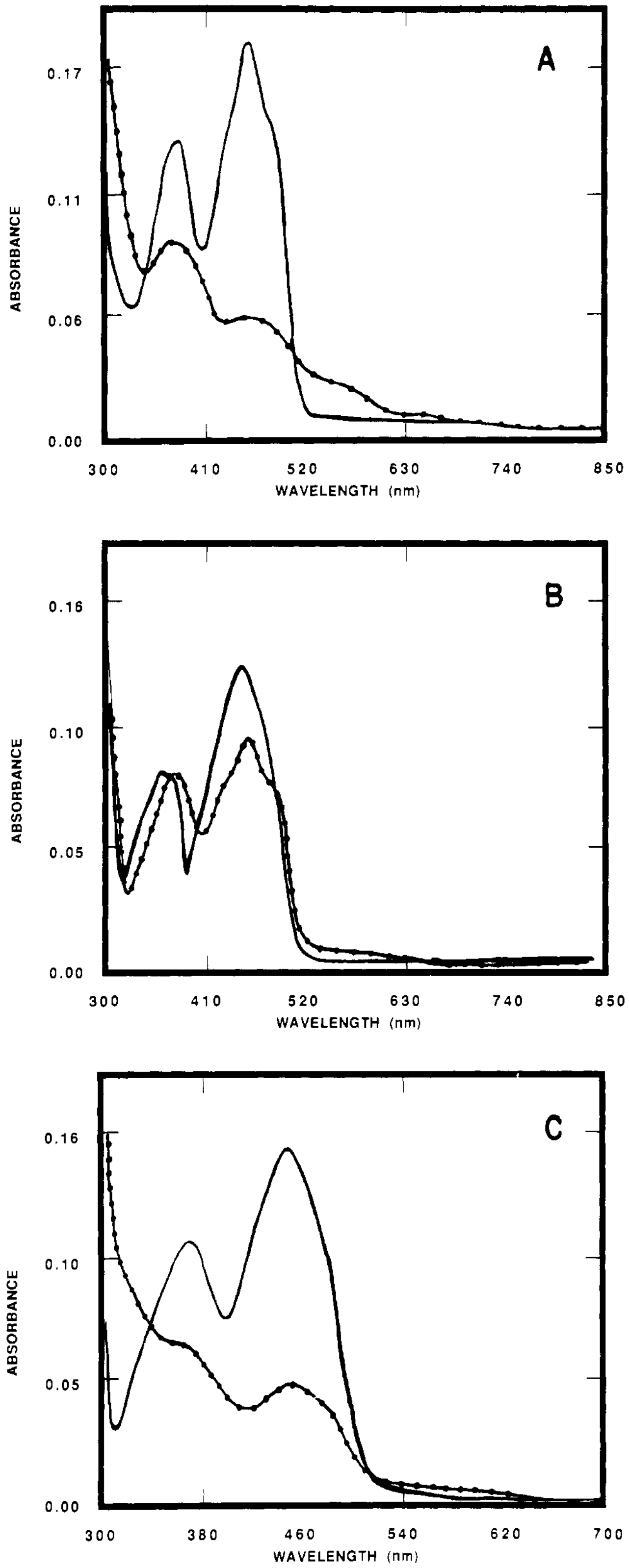
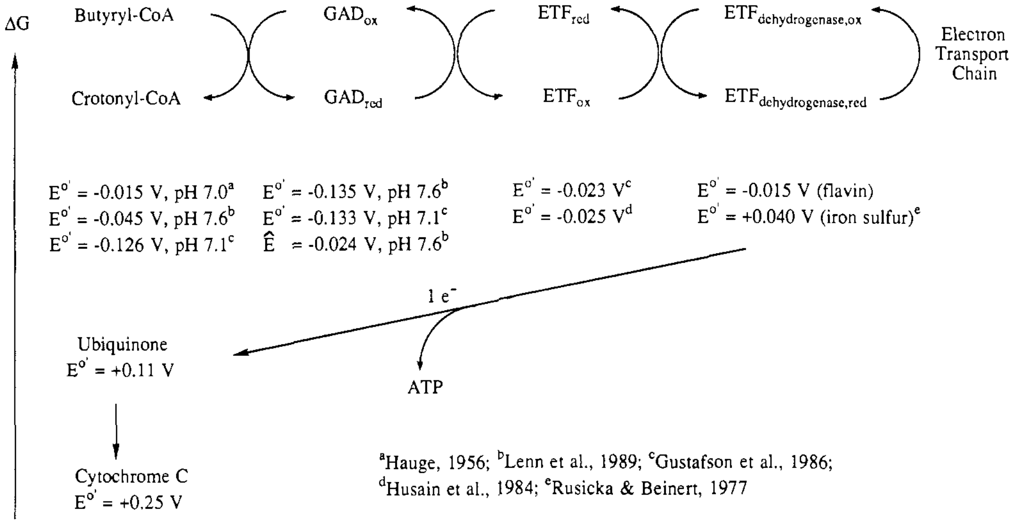


FIGURE 2: Spectra from the potentiometric measurements of product- and substrate-bound GAD (8.3–10 μM) which has been mixed with a 1:1 ratio of the oxidized/reduced redox couple (final concentration 300 μM). Spectra have been corrected for IDS (5 μM) and PYC (20 μM) contributions for clarity: (A) *trans*-2-octenoyl-CoA/octanoyl-CoA; (B) *trans*-2-hexadecenoyl-CoA/palmitoyl-CoA; (C) crotonyl-CoA/butyryl-CoA [GAD_{ox} (—); GAD bound at equilibrium (●–●)].

Analysis of the crotonyl-CoA/butyryl-CoA couple was somewhat complicated by both the intrinsic hydratase activity and a "slow reaction" that does not occur with the other two

Scheme II: Isopotential of Delivery of Electrons through the Fatty Acid Oxidation Cycle and into the Electron-Transport Chain



substrates examined (Schopfer et al., 1988). The hydratase activity catalyzed the slow turnover of crotonyl-CoA to β-hydroxybutyryl-CoA, which is indicated by a slow negative drift in potential during long equilibration times. Additionally, the slow reaction of butyryl-CoA with GAD has a $t_{1/2} = 30$ min and results in bleaching of the flavin spectrum without a concomitant flavin reduction. The exact nature of this process is unknown, leaving a degree of ambiguity when trying to quantify the concentration of oxidized and reduced GAD which is substrate bound in these experiments. Schopfer et al. have proposed that the end point of the slow phase is a ligand complex of fully reduced GAD. However, equilibration was quite fast with this couple as indicated by an equal concentration of $GAD_{red}{\cdot}P$ at both 15 and 120 min after tip in of substrates and a ΔE_m of only 3 mV over this time period. Thus, by use of molar absorptivities at 450 nm of $GAD_{ox}{\cdot}P$, $GAD_{ox}{\cdot}S = 13.8\ mM^{-1}$, and $GAD_{red}{\cdot}P = 2.95\ mM^{-1}$ (560 nm), an $\hat{E} = -0.030$ V was obtained.

When any of the GAD–acyl-CoA equilibrium solutions were perturbed by the addition of either oxidative or reductive charge, it would return to within 2 mV of its equilibrium potential within 2 h. Addition of oxygen to the system resulted in reoxidation of the flavin spectrum. Due to substrate and product binding, the reoxidized spectrum was always red shifted and showed a lower extinction coefficient than ligand-free GAD. No IDS reduction was observed with any of the substrates, and PYC was nearly 50% reduced at equilibrium.

DISCUSSION

It is apparent from the results shown in Figure 1 that the straight-chain acyl-CoA thioesters are indeed isopotential with only a 0.007-V range from the C-4 to C-16 chain length. When the values obtained by Stankovich and Soltysik (1987) and Hauge (1955) for the crotonyl-CoA/butyryl-CoA couple are adjusted for pH differences, they are within 0.001 V of the $E^{\circ\prime}$ values obtained herein. Had the CoA thioester exhibited a significantly more negative $E^{\circ\prime}$ such as that reported by Gustafson et al. (1986), a complete reduction of PYC and partial reduction of IDS would have been seen due to the large excess of the redox couple over dye. The lack of any IDS reduction and a ratio of oxidized/reduced PYC similar to that of the product/substrate ratio argue against such negative potentials.

Our data on CoA couples differ markedly from the values obtained by Gustafson et al. The fact that the enzyme by Gustafson et al. was isolated from pig liver should not be a complication factor since we have obtained similar values for the butyryl-CoA/crotonyl-CoA couple using acyl-CoA dehydrogenase from *M. elsdenii* and pig kidney. One possible explanation for this difference is that Gustafson et al. did not measure the potential of the crotonyl-CoA/butyryl-CoA couple directly as was done in the present work. Rather, the GAD was equilibrated with the FA-CoA/FP-CoA couple, and an $E^{\circ\prime}$ value for this couple was obtained from the equilibrium position of GAD in the presence of FA-CoA/FP-CoA (Gustafson et al., 1986). For these calculations it was assumed that the potential of GAD did not change in the presence of substrate and product. By use of this $E^{\circ\prime}$ value and the K_{eq} obtained from the incubation of GAD with FP-CoA and crotonyl-CoA, a potential for the crotonyl-CoA/butyryl-CoA couple was determined (McFarland et al., 1982). Such indirect measurements have capacity for error, some of which have been discussed previously (Stankovich & Soltysik, 1987).

Binding of the CoA thioester to GAD resulted in a positive shift of the GAD midpoint potential of 0.070–0.095 V for the acyl-CoA thioesters studied. The agreement in $E^{\circ\prime}$ values for the dyes PYC and IDS and the ligand-free GAD between these experiments and those of Gustafson et al. (1986) confirm that the potential shifts observed upon substrate and product binding are real and not merely a result of cell methodology. Precedence for such a potential shift in GAD was observed from the interaction of GAD with its in vivo reoxidizing agent ETF. The $E^{\circ\prime}$ values for these two enzymes are −0.136 and −0.023 V, respectively (this work; Husain et al., 1984; Gustafson et al., 1986). Gorelick et al. (1985) observed that when the two enzymes are mixed and treated with dithionite, complete reduction of ETF occurs prior to GAD reduction. However, the addition of substrate results in an equilibrium between GAD·P and ETF red anionic semiquinone ($E^{\circ\prime}_{semiquinone} = +0.004$ V; Husain et al., 1984). If GAD·P maintained an $E^{\circ\prime}$ equal to that of the ligand-free GAD, then complete reduction of ETF should have been observed.

These studies show that GAD, like its bacterial counterpart BCD, relies on binding of the substrate to initiate electron transfer. It is not surprising that this is the case since all of the straight-chain acyl-CoA dehydrogenases, regardless of their source, share a common mechanism (Schopfer et al., 1988). This reinforces the theory that the evolution of these enzymes maintained the catalytic machinery required for a specific reaction while functional groups responsible for determining substrate specificity changed (Manstein et al., 1988). The observed shift in the $E^{\circ\prime}$ of GAD and BCD upon binding of substrate and product confirms earlier hypotheses that the dehydrogenases put a significant amount of energy into the binding and ionization of CoA thioesters with no free energy minimum along the dehydrogenase reaction coordinate (Lau et al., 1988). This lowering of transition-state energy during enzyme turnover gives an isopotential delivery of electrons into the electron-transport chain. Thus, these enzymes function similarly to a conducting wire, passing electrons from fatty acyl-CoA to the electron-transport chain where this energy is captured as ATP. As shown in Scheme II, a large drop in energy is not seen until it is coupled to the formation of ATP, which is more energetically efficient than if a loss of energy from this electron transfer were to occur earlier in the cycle. Thus, the transfer of electrons from acyl-CoA thioesters into the electron-transport chain is an important instance of enzymatic thermodynamic control and regulation in an essential energy production pathway.

Electrochemistry has provided a valuable insight into the energetics and regulation of fatty acid metabolism by enabling us to measure potential shifts in the presence of substrates and products. At this point we must leave the potential shifts in terms of $\hat{E}$ or conditional potentials. We cannot deduce the individual binding constants on the basis of the observed potential shifts because of the complex mixture of enzyme species present. This system may contain three forms of reduced enzyme species, i.e., the charge-transfer complex, the Michaelis complex between reduced enzyme and product, and the complex between reduced enzyme and substrate. They are not discernible spectrally. In order to differentiate these species and thus to obtain binding constants, we have to work on a simple system. Therefore, future studies of this process will make use of substrate and product analogues. Analogues that do not undergo complete turnover but bind to the enzyme would allow both determination of binding constants and investigation into what binding processes are crucial to the potential shift and regulation of acyl-CoA dehydrogenases.

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