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Oxidation–Reduction Potentials of Butyryl-CoA Dehydrogenase†

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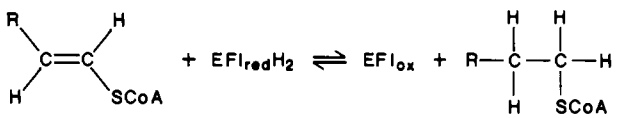
ABSTRACT: In order to obtain butyryl-CoA dehydrogenase from *Megasphaera elsdenii* in pure enough form to perform redox studies, the existing purification procedures first had to be modified and clarified [Engel, P. (1981) *Methods Enzymol. 71*, 359–366]. These modifications are described, and the previously unpublished spectral properties of the electrophoretically pure CoA-free butyryl-CoA dehydrogenase are presented. In our spectral reductive titration of pure enzyme, we show that although blue neutral flavin radical is stabilized in nonquantitative amounts in dithionite titrations (19%) or in electrochemical reductions mediated by methylviologen (5%), it is not thermodynamically stabilized; therefore, only a midpoint potential for butyryl-CoA dehydrogenase is obtained. The electron-transfer behavior from pH 5.5 to pH 7.0 indicates reversible two-electron transfer accompanied by one proton:

$$EFl_{ox} + 2e^- + H^+ = EFl_{red}H^- \quad E_{m7} = -0.079 \text{ V vs. SHE}$$

where EFl_{ox} is oxidized butyryl-CoA dehydrogenase, $EFl_{red}H^-$ is two electron reduced enzyme, and E_{m7} is the midpoint potential at pH 7.0 at 25 °C. Redox data and activity data both indicate that the enzyme loses activity rapidly at pH values above 7.0. The E_{m7} of the butyryl-CoA dehydrogenase is 40 mV positive of the E_{m7} of the butyryl-CoA/crotonyl-CoA couple [Gustafson, W. G., Feinberg, B. A., & McFarland, J. T. (1986) *J. Biol. Chem. 261*, 7733–7741]. Binding of substrate analogue acetoacetyl-CoA caused the potential of butyryl-CoA dehydrogenase to shift 100 mV negative of the free enzyme. The negative shift in potential makes electron transfer from enzyme to substrate more probable, which is consistent with the direction of electron transfer in the bacterial system. Our work suggests that substrate binding may cause the potentials of the mammalian and bacterial fatty acyl-CoA dehydrogenases to shift in different directions in accord with the opposing directions of electron transfer in the two kinds of organisms.

Butyryl-CoA dehydrogenase (EC 1.3.99.2) from *Megasphaera elsdenii* catalyzes the two electron transfer reaction in fatty acid metabolism shown below. The substrates in the



reaction are the same as for the mammalian system, but the direction of the electron transfer is reversed, with the bacterial

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enzyme producing the reduced butyryl-CoA[1] as shown (Williamson, 1983; Engel, 1981). We are interested in studying the electron-transfer properties of the bacterial enzyme. We hope the properties of the bacterial system will give us insights into the mammalian enzyme, which is more difficult to purify (Engel, 1981), and offer comparison between these two systems.

Butyryl-CoA dehydrogenase from the bacterial source *M. elsdenii* offers many advantages over the mammalian system in ease of preparation. First, the bacteria has only one fatty acyl-CoA dehydrogenase present (BCD), and second, the enzyme comprises about 2% of the dry weight of the bacteria (Engel, 1981; Engel & Massey, 1971a). Both of these factors allow for a high yield of protein without contamination by other acyl-CoA dehydrogenases, thus greatly decreasing time and effort spent in the purification process. The procedure for the purification of butyryl-CoA dehydrogenase has been published (Engel & Massey, 1971a; Engel, 1981); however, upon implementing this procedure, one finds that the enzyme exhibits a long wavelength absorbance centered at 710 nm, which makes the enzyme appear green, in contrast to the normal yellow color of flavoproteins. The fraction of the enzyme in the complexed form varies from preparation to preparation (Williamson & Engel, 1981), which makes the spectral properties of the enzyme variable. This characteristic long wavelength absorbance is due to a $CoASS^-$–BCD complex (Williamson et al., 1982). The procedure for removing this ligand to give a homogeneous sample of $CoASS^-$ - and CoA-free enzyme has been described (Williamson & Engel, 1982).

Because we can quantitatively obtain the ligand-free form of BCD, we can now take advantage of the high yield and comparative ease of purification and use BCD as a model system to rigorously study the electron-transfer process in fatty acid metabolism.

Most of the work done on BCD until now has centered on its unusual spectral properties. However, Massey and Engel have reductively titrated the yellow enzyme containing variable amounts of CoA with dithionite, obtaining a two-electron transfer to FAD (Engel & Massey, 1971a). No radical formation was seen on reductive titration with dithionite or EDTA–light; however, on reoxidation with oxygen, small amounts of $EFlH^\bullet$ were observed (Engel & Massey, 1971a). We wanted to more fully measure the redox properties of the CoA-free BCD using the spectroelectrochemical techniques previously developed (Stankovich, 1980; Stankovich & Fox, 1983). By use of these methods, coulometric reductions, dithionite reductions, and reductive potentiometric titrations have been done, providing interesting insights into the contribution this enzyme may make to fatty acid metabolism.

Materials and Methods

Butyryl-CoA dehydrogenase was isolated from *M. elsdenii* by using Engel's method (Engel, 1981) with the modifications described under Results. The *M. elsdenii* cultures used were the generous gift of Dr. Vincent Massey and Dr. Larry Schopfer at the University of Michigan, Ann Arbor, MI. The removal of the $CoASS^-$ to give yellow CoA-free BCD was done according to published methods (Williamson & Engel, 1982), but the procedure was modified (see Results) to avoid irreversible binding of the enzyme to thiopropyl-Sepharose 6B, as supplied by Pharmacia Fine chemicals.

The purity of the enzyme was checked by using its distinct spectral properties (Engel & Massey, 1971a), enzyme assay, and later, SDS gel electrophoresis. The assay was performed as previously described by Engel (Engel, 1981; Engel & Massey, 1971a); 2,6-dichlorophenolindophenol was purchased from ICN Pharmaceuticals. The dye was not very soluble, so it was filtered on a sintered-glass funnel 4 times before use.[2] Butyryl-CoA and phenazine methosulfate were purchased from Sigma.

SDS gel electrophoresis was performed with an LKB electrophoretic unit and power supply. Bis(acrylamide), acrylamide, Trizma base, bromphenol blue, and the molecular weight standard were purchased from Sigma.

The enzyme passed through the thiopropyl-Sepharose 6B column was assayed for the complete removal of CoASH by incubation with sodium sulfide (Williamson et al., 1982).

Various mediator dyes were used throughout the three different types of titrations. These dyes include MV^{2+}, purchased from British Drug House, Poole, England; PYC, the decomposition product of phenazine methosulfate (McIlwain, 1937); IDS, purchased from Matheson, Coleman, and Bell; 2-H1,4NQ, purchased from Eastman Kodak; and PHE, purchased from British Drug House. 8-Chlororiboflavin is the gift of Dr. Lambooy, University of Maryland.

Three types of titrations have been done in this work: coulometric titration, dithionite reductions, and potentiometric titration. Coulometric titrations were done as described, using MV^{2+} as the mediator titrant (Stankovich, 1980; Stankovich & Fox, 1983). Dithionite titrations were performed as previously described (Foust, 1969). Approximately 2 mM dithionite in 0.05 M pyrophosphate buffer, pH 8.0, was first standardized with lumiflavin acetate, which was the gift of Dr. Sandro Ghisla, University of Konstanz, Konstanz, West Germany. The standardized dithionite was then used to reduce BCD in an anaerobic spectrophotometric cell. The experiments were run at pH 7.0, 0.1 M potassium phosphate, at 25 °C.

The methodology of the potentiometric titrations has been described previously (Stankovich, 1980; Stankovich & Fox, 1983). Both electrochemical and dithionite reduction were used in the potentiometric titrations, with identical results. Initially, four mediator dyes were present to determine an approximate midpoint potential. This mixture of dyes gives a potential range of −0.015 to −0.240 V vs. SHE. The dyes were used in the following concentrations: 2 μM PYC, 2 μM IDS, 3 μM 2-H1,4NQ, and 2 μM PHE. This mixture of dyes plus enzyme, about 10 μM, gave us an estimate of the midpoint potential. With the potential obtained in the preliminary experiment, the mixture of dyes was then narrowed to the two with potentials near that of the enzyme. PYC, which has a midpoint potential ($E^{\circ 1}$) of −0.015 V, and IDS, which has an $E^{\circ 1}$ of −0.111 V vs. SHE at pH 7.0 in 0.1 M potassium phosphate buffer, were then used. Each of these dyes was titrated in the absence of enzyme for two reasons: first, so that spectral corrections for the contributions of the dye to the

[1] Abbreviations: BCD, butyryl-CoA dehydrogenase; EFl_{ox}, oxidized form of butyryl-CoA dehydrogenase; $EFlH^\bullet$, blue neutral radical of butyryl-CoA dehydrogenase; $EFl_{red}H^-$, two electron reduced anion forms of butyryl-CoA dehydrogenase; $CoASS^-$, CoA persulfide; SDS, sodium dodecyl sulfate; MV^{2+}, methylviologen; PYC, pyocyanine; IDS, indigodisulfonate; 2-H1,4NQ, 2-hydroxy-1,4-naphthoquinone; PHE, phenosafranin; GCD, glutaryl-CoA dehydrogenase; ETF, electron-transferring flavoprotein; EDTA, ethylenediaminetetraacetic acid; EPR, electron spin resonance; FAD, flavin adenine dinucleotide; E_m, midpoint potential (this is the interaction of the Nernst plot of the system; it is used instead of $E^{\circ 1}$ because we cannot resolve this into individual electron transfers); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[2] Added in proof: Results using dichlorophenolindophenol from General Biochemicals are better.

spectra could be determined, and second, so that a check could be made to ensure that the dye was not binding to enzyme. Any deviation of either the redox or spectral properties of the mediator dyes in the presence of enzyme may indicate binding of dye to enzyme. No such changes were seen during the course of this study, indicating no enzyme–dye interactions.

The ratio of dye to enzyme was 1:6 or 1:10, depending on the molar absorptivity of the dye used. It is necessary to select dyes that are in the potential region needed to assure thermodynamic equilibrium with the enzyme, and they must also interfere minimally with the enzyme spectrum. Two hours of equilibration time was allowed between points and a potential drift of less than 1 mV/5 min was allowed. These parameters were used to ensure that thermodynamic equilibrium was established.

Spectrophotometric measurements were made with either a Varian 210 or 219 spectrophotometer, equipped with a thermostated cell holder and interfaced to an Apple II^+ or IIe computer. A Princeton Applied Research 173 potentiostat and 179 digital coulometer were used in the electrochemical experiments. Fluorescence measurements were performed with a Varian SF-330 fluorometer.

EPR Measurements were made on a Varian 109 spectrometer in the laboratory of Dr. John Lipscomb at the University of Minnesota. A modified procedure of Beinert (Beinert et al., 1978) was used to maintain anaerobicity. Experiments were done in 0.1 M potassium phosphate, pH 7.0, with an enzyme concentration of 32.1 μM. All reduction steps were carried out under a nitrogen atmosphere. The instrument conditions were: temperature, 12.2 K; microwave power, 0.2 μW; microwave frequency, 9.22 GHz; modulation amplitude, 10 G; modulation frequency, 100 Hz; g-factor, 2.01; 1 mM $CuClO_4$ standard. The visible spectrum was recorded in the EPR tube, which had been previously standardized to determine the cell path length, on a Hewlett-Packard 8451A diode array spectrophotometer.

RESULTS

Enzyme Purification. There is a published method (Engel, 1981) for purifying BCD and one for removing the tightly bound $CoASS^-$ ligand, yielding free enzyme (Williamson & Engel, 1982); however, two serious problems develop if these methods are not modified. First, although the enzyme at step three is stated to be sufficiently pure for most purposes (Engel, 1981), we have found that the BCD thus prepared showed three bands on SDS–PAGE: a very light band at about 58 000 daltons, a strong BCD band at 43 000 daltons, and a band of low intensity at less than molecular weight 20 000. The addition of a Sephadex G100 or Sephacryl S200 column (Engel preparation, optional step 4) removes these impurities. The addition of the gel chromatography and following the preparation with SDS–PAGE are essential.

Second, there was a serious problem with the thiopropyl-Sepharose 6B column. According to Williamson and Engel (1982), the material is supplied ready to use by Pharmacia; however, we found that BCD binds irreversibly to fresh column material. This irreversible binding was observed for two different lots of column material bought one year apart. In each case, the BCD could only be removed under strong reducing conditions, e.g., mercaptoethanol, but then readily denatured. After this initial irreversible adsorption of protein and elution with mercaptoethanol, the column was recycled according to the method of Williamson and Engel (1982), which is essentially the recycling procedure recommended by Pharmacia; the column worked well for a year with recycling between preparations. All loss of protein could be prevented if the thiopropyl-Sepharose was precycled before its initial use.

The BCD fractions collected from the thiopropyl-Sepharose 6B column were assayed spectrally for the presence of the 2-thiopyridone leaving group of the affinity column. 2-Thiopyridone has an ϵ at 343 nm of 0.08 mM^{-1} cm^{-1} (Williamson & Engel, 1982).

In summary, our recommended procedure that yielded 95% pure enzyme follows Engel's procedure through the Sephadex G100 step (step 4). This enzyme is dialyzed against dithionite overnight and then against buffer for 3 h; it is then applied to thiopropyl-Sepharose that has been precycled. Enzyme that is purified in this manner exhibits a single band on SDS–PAGE and is at least 95% free of the $CoASS^-$ ligand, as judged by the assay for $CoASS^-$ (Williamson et al., 1982). Spectral properties of the purified enzyme are given by the absorbance ratios: 1.0:0.69:5.6 at 430, 365, and 266 nm, respectively. A complete description of these spectral values has not been published previously; however, the 260:430 absorbance ratio was noted to decrease significantly with the use of the thiopropyl-Sepharose column (Williamson & Engel, 1981). The specific activity of the pure BCD is between 200 and 300 $\mu M\ min^{-1}$ (μM enzyme)$^{-1}$.

We estimate at least a 10% impurity in the less pure enzyme after step three. Experiments performed on the less pure enzyme sample yielded quite different results from those performed on the pure enzyme in all three types of titrations described below. Thus, the 10% impurity must have interfered strongly with the properties of BCD, leading us to conclude that the impurity must have spectral and redox properties similar to those of BCD or that it binds BCD. This impurity is likely to be a low molecular weight flavoprotein that stabilizes a large amount of blue neutral radical itself, and the potential of the first electron transferred to the impurity is necessarily near that of BCD. Flavodoxin fits this description (Mayhew & Massey, 1969), and flavodoxin is one of the enzymes occurring in *M. elsdenii* that must be separated from BCD. We will show that the removal of this small molecular weight impurity is essential to reliable electrochemical experiments.

We estimate the percent of low molecular weight impurity in the following way. The BCD with the impurity stabilized 20–24% more neutral radical than the purified enzyme in all three kinds of experiments (see below). We assumed that the impurity stabilized 100% radical and that the molecular weight of the impurity was 15 000/flavin, as opposed to the molecular weight of pure enzyme, which is 43 000/flavin. This corresponds to about 10% on the basis of protein weight for the impurity. This fits with our spectral estimates of the amount of Coomassie blue dye bound to each protein band in SDS–PAGE.

In the results of the titrations given below, the preliminary results on less pure enzyme refer to enzyme that had been passed through the thiopropyl-Sepharose 6B column but not through Sephadex G100. The pure enzyme refers to 95–100% pure enzyme that had been passed through both Sephadex G100 and thiopropyl-Sepharose 6B.

Preliminary Results on Impure Enzyme. The combined spectral and EPR experiment described previously, which was used to determine the molar absorptivity of the $EFlH^\bullet$ at 580 nm, was done only on impure enzyme. The molar absorptivity of radical at 580 nm is estimated to be 5600 M^{-1} cm^{-1}, in good agreement with the value for general acyl-CoA dehydrogenase (Thorpe et al., 1979). This was not redetermined for the pure enzyme, because in potentiometric titrations, the pure enzyme does not stabilize neutral radical. Therefore, rigorous values

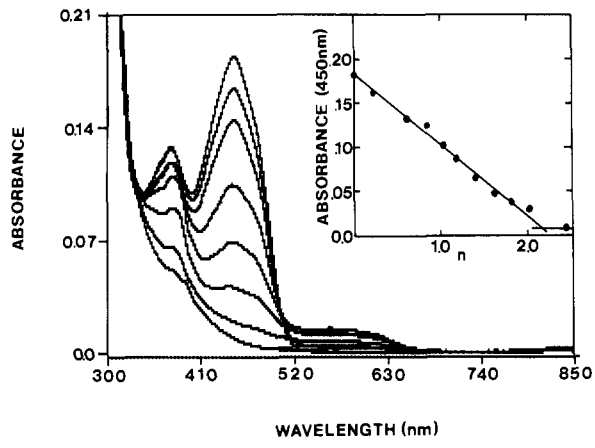


FIGURE 1: Dithionite titration of 1.27×10^{-5} butyryl-CoA dehydrogenase in 0.10 M potassium phosphate buffer, pH 7.0 at 25 °C. Inset is a plot of A_{450} vs. the number of charge equivalents added.

of molar absorptivity were not needed to calculate the redox potential values. The estimated molar absorptivity was used only to estimate the percent radical kinetically stabilized in dithionite titrations and coulometric titrations for qualitative comparative purposes (see below). Thirty-nine percent EFlH• is stabilized in a dithionite titration. Although dithionite was capable of reducing EFlH•, the electrochemically generated methylviologen radical was not capable of reducing the EFlH• produced upon reduction.

The potentiometric experiments that were run in the presence of four mediator dyes covering a broad potential range were only done with the impure enzyme. They were not repeated on the pure enzyme because the potential region identified for the impure enzyme is similar to that of the pure enzyme. No binding of dyes to the pure or impure enzyme was seen. The midpoint potential of the less pure enzyme is −0.106 V vs. SHE and has a Nernst slope equaling 0.066 V in the reductive direction at pH 7.0. Oxidative data gave a midpoint potential of −0.111 V vs. SHE and a Nernst slope of 0.044 V. Approximately 24% blue neutral radical was stabilized during this potentiometric titration.

Pure Enzyme: Coulometric Titration. Coulometric titration can provide both qualitative and quantitative information about an enzyme and its redox properties. Distinct spectral characteristics as well as the amount and type of flavin radical species present may be determined. A very small amount of EFlH• is stabilized in this type of titration. Using the molar absorptivity from the EPR experiment, we calculated that 5% EFlH• is the maximum amount stabilized at the midpoint in coulometric titrations at pH 7. Two electrons per FAD molecule are required to fully reduce the enzyme, which indicates that there are no other redox active sties present in the BCD. From the fully reduced spectrum, we calculate the molar absorptivity of 1200 M^{-1} cM^{-1} for the $EFl_{red}H^-$ at 450 nm; MV^{2+} is perfectly capable of fully reducing this enzyme, in marked contrast to the coulometric titration of the impure enzyme.

Dithionite Titration. In an effort to kinetically stabilize a large amount of EFlH•, a dithionite titration was performed at pH 7, with the results shown in Figure 1. Approximately 19% EFlH• is seen. Confirming these results with electrochemical reduction, two electrons per FAD molecule are required to fully reduce the butyryl-CoA dehydrogenase. The results here are in contrast to previous results where no EFlH• was seen upon dithionite reduction (Engel & Massey, 1971a), but are similar to those for a dithionite titration performed by Williamson.[3]

In the dithionite titration of the enzyme, the absorbance at 380 nm does not change for the first two reduction points in the titration. A possible explanation for this behavior involves the following points: First, a maximum of 20% EFlH• is formed in this titration. The reduction profile (plot of absorbance vs. percent reduced) shows that initially most of the charge goes to form radical, with little initial formation of the two electron reduced form. Second, the molar absorptivities of the oxidized enzyme and enzyme radical are estimated to be equal (10000 M^{-1} cm^{-1}) at 380 nm. The molar absorptivity of the oxidized enzyme is calculated from the spectrum of oxidized enzyme and the known molar absorptivity of the oxidized enzyme at 450 nm (14400 M^{-1} cm^{-1}) (Williamson & Engel, 1982). The molar absorptivity of the radical is about 5600 M^{-1} cm^{-1} at 580 nm (this paper), which is equal to the molar absorptivity of Shethna flavoprotein at 580 nm (Barman & Tollin, 1972). The molar absorptiviity at 380 nm is estimated to be about 10000 for Shethna protein, from a published spectrum (Edmondson & Tollin, 1971). Therefore, 10000 M^{-1} cm^{-1} is a reasonable estimate for molar absorptivity of EFlH•. Since the molar absorptivity of oxidized enzyme and that of EFlH• are the same at 380 nm, one would not expect much change in absorbance until substantial amounts of the two electron reduced enzyme are produced.

Potentiometric Titration. We determined the pH dependence of the electron transfer. Therefore, we measured the redox potentials over a broad pH range, 5.5–8.5. The behavior at pH 7.0 will be presented first since the most rigorous tests for reversibility were done at this pH. The results of electrochemical reduction and dithionite reduction were the same. Figure 2 shows the reductive titration of BCD with mediators PYC and IDS. The enzyme and the dye are being reduced concurrently. No thermodynamic stabilization of the blue radical is seen when spectral subtraction of IDS is done. An E_m of −0.079 V vs. SHE is seen both in the reductive and the oxidative directions. Plots of measured potential vs. logarithm $(EFl_{ox}/EFl_{red}H^-)$ give a slope of 0.038 V in the oxidative direction and a slope of 0.039 V in the reductive direction, both of which are near the Nernstian value of 0.030 V for a two-electron transfer. These data clearly show the complete reversibility of this electron transfer. The E_m of the IDS is the same in this experiment as in the absence of the enzyme, indicating that no binding of dye to the enzyme is occurring.

A potentiometric titration of BCD at pH 6, 0.1 M potassium phosphate buffer, at 25 °C, and in the presence of IDS as redox mediator gives an E_m of −0.049 V vs. SHE. The potential vs. logarithm $(EFl_{ox}/EFl_{red}H^-)$ plot gives a Nernst slope of 0.035 V, which compares quite well to the theoretical slope of 0.030 V. A potentiometric titration of BCD at pH 5.5 in 0.10 M sodium acetate in the presence of IDS redox mediator gives an E_m of −0.035 V vs. SHE. The potential vs. logarithm $(EFl_{ox}/EFl_{red}H^-)$ plot gives a Nernst slope of 0.030 V, which is identical with the theoretical slope. Looking at the potential/pH dependence at these three values, we have a 0.030 mV/pH unit change, indicating that one H^+ is transferred with two electrons at pH values of 7 and below. There is no thermodynamic stabilization of EFlH• at these pH values.

Titrations of BCD at more basic pH values yield unusual results. At pH 7.8 in 0.1 M potassium phosphate at 25 °C, one sees an approximate E_m of −0.200 V vs. SHE. At pH 8.5 in 0.05 M pyrophosphate at 25 °C, one gets an approximate E_m of −0.272 V vs. SHE. The enzyme undergoes dramatic spectral changes at these higher pH values. These E_m values are only approximate because there are no mediator dyes

[3] Dr. Gary Williamson, personal communication.

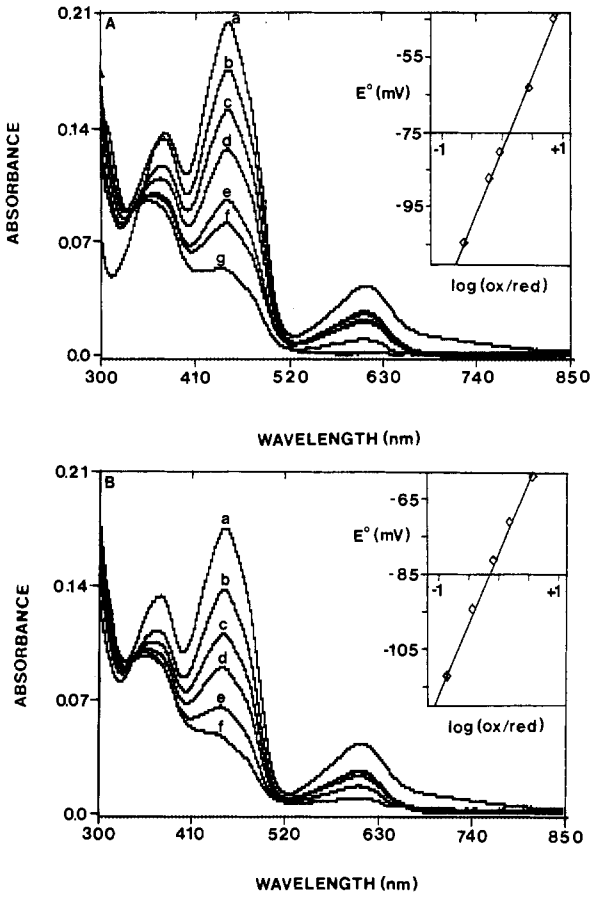


FIGURE 2: Potentiometric titration of 1.27×10^{-5} butyrl-CoA dehydrogenase in 0.10 M potassium phosphate buffer, pH 7.0 at 25 °C. Redox indicators are pyocyanine (3 μM) and indigodisulfonate (2 μM); redox mediator 2,6-dichlorophenolindophenol is present in 0.4 μM concentration. (A) Reductive data; 1 mM sodium dithionite is the titrant. (a) Initial spectrum—oxidized enzyme with no dyes; (b) oxidized BCD plus dyes; (c) reductive point, measured potential −0.045 V vs. SHE; (d) measured potential −0.063 V vs. SHE; (e) measured potential −0.080 V; (f) measured potential −0.087 V; (g) measured potential −0.104 V. Inset is the Nernst plot of the reductive data. (B) Oxidative data obtained by electrochemically pulsing the potential positive after each reductive point. (a) Start of the experiment—oxidized BCD plus dyes; (b) oxidative point, measured potential −0.059 V vs. SHE; (c) oxidative point, measured potential −0.071 V; (d) oxidative point, measured potential −0.081 V; (e) oxidative point, measured potential −0.094 V; (f) oxidative point, measured potential −0.112 V. Inset is the Nernst plot of the oxidative data.

present in the system that would be in equilibrium with the enzyme at these negative potentials.

In order to understand the electrochemical results, 10 μM BCD was incubated at 25 °C at the five pH values used in the electrochemical experiments. Enzyme activity was assayed every hour for 6 h, and then after 22 h. BCD was most stable at pH 6.0, losing no activity after 22 h. At pH 5.3 and 7.0, enzyme activity remained constant over the 6-h period, but after 22 h, the activity was 81% and 76%, respectively, of the original activity. At pH 7.8 BCD lost 50% of its activity in 3.4 h; after 6 h, 33% of the activity remained; after 22 h, 11% of the activity remained. However, at pH 8.5, after 1.5 h, the activity was 27% of the original; after 4.5 h, it was less than 9% of the initial activity. Thus at pH values greater than 7.0, BCD loses activity rapidly with respect to the time scale of the electrochemical experiment. At least 2 h are required to add mediators individually to the enzyme, record the spectra, and make the enzyme anaerobic. If 2 h of equilibration are required per point in the titration, the first point is taken when the BCD has been in the buffer 4 h. At pH 7.8 and 8.5, the

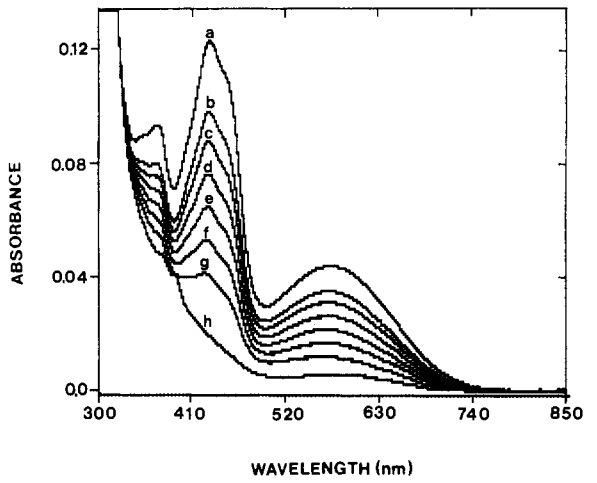


FIGURE 3: BCD coulometric titration with acetoacetyl-CoA inhibitor, 0.1 M potassium phosphate buffer, pH 7.0, 25 °C, 9.6 μM BCD, 100 μM acetoacetyl-CoA, and 100 μM MV^{2+}. (a) Initial spectrum after degassing cell, $n = 0$; (b) $n = 0.053$; (c) $n = 0.80$; (d) $n = 1.09$; (e) $n = 1.36$; (f) $n = 1.63$; (g) $n = 1.91$; (h) $n = 2.3$, end point.

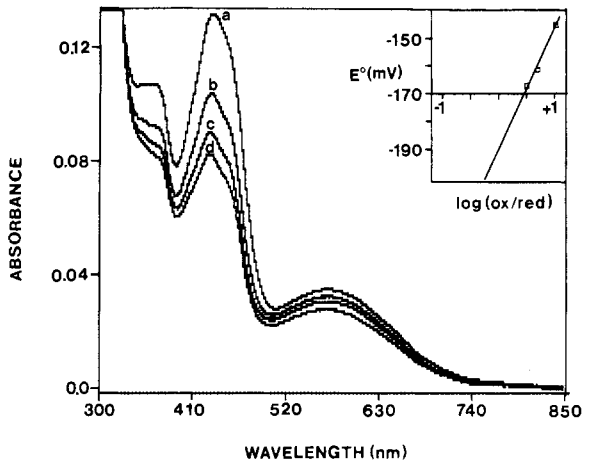


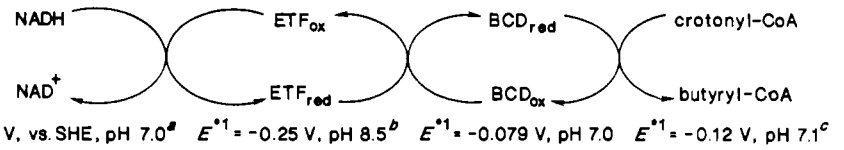
FIGURE 4: BCD potentiometric titration with acetoacetyl-CoA inhibitor and 8-chlororiboflavin as redox indicator, 0.1 M potassium phosphate buffer, pH 7.0, 25 °C, 7.3 μM BCD, 100 μM acetoacetyl-CoA, 3.5 μM 8-chlororiboflavin, and 100 μM MV^{2+}. (a) Initial spectrum after degassing cell; (b) $E = -145$ mV; (c) $E = -161$ mV; (d) $E = -167$ mV.

BCD has undergone significant inactivation.

It was possible that the inactivation of BCD was accompanied by partial loss of FAD, which could itself serve as a redox indicator at the relatively negative potentials observed. This was tested by measuring the fluorescence of 10 μM BCD incubated at pH 8.5 at 25 °C as a function of time. The initial fluorescence reading taken 10 min after the start of incubation showed that the BCD itself was slightly fluorescent. After 2 h, the fluorescence reading increased, indicating that 2 μM FAD had dissociated from the BCD. This indicated that small amounts of FAD may have dissociated from the BCD as it lost activity; these small amounts of FAD could serve as redox indicators poising the electrode potential at the relatively negative values observed here.

Acetoacetyl-CoA-Bound BCD. The inhibitor acetoacetyl-CoA is similar in structure to crotonyl-CoA, which is the substrate for bacterial BCD (Engel & Massey, 1971b). The conditions for the experiment are from Williamson (1983). Figure 3 (a) shows that acetoacetyl-CoA binds to EFl_{ox}, because a distinctive charge-transfer complex is formed. Figure 3 shows that as acetoacetyl-CoA-bound BCD is electrochemically reduced in a coulometric titration, the spectral properties

Scheme I



[a] Underwood & Burnett, 1973. [b] C. Pace, unpublished results. [c] Gustafson et al., 1986.

of the charge-transfer complex decrease as the 450-nm flavin band decreases, indicating no charge-transfer complex is occurring as the BCD is reduced by a two-electron transfer. Individual potentiometric titrations were performed, using IDS, 8-chlororiboflavin, and ruthenium hexaammine as redox indicators. None of the redox indicators precisely matched the E_m of the inhibitor-bound BCD; however, all three experiments gave an E_m of −0.180 mV for the acetoacetyl-CoA-bound BCD at pH 7.0. Figure 4 shows the titration of acetoacetyl-CoA-bound BCD with 8-chlororiboflavin as the redox indicator. There are only three experimental points taken because the E_m of 8-chlororiboflavin does not exactly match the E_m of inhibitor-bound BCD. Therefore, it acts as a redox indicator and mediator at potential values near its own E_m of −0.150 V at pH 7.0. The insert in the figure shows the Nernst plot, which gives an intersection indicating an E_m of −0.180 V with a 30-mV slope. Thus the binding of acetoacetyl-CoA shifts the potential of BCD negative by 100 mV with respect to free BCD.

Discussion

Experiments on the pure enzyme encompassed a pH range of 5.5–8.5. The EFlH$^\bullet$ stabilized in dithionite titrations (19%) and coulometric titrations (5%) must be kinetically stabilized, since during potentiometric titrations where the thermodynamic equilibrium is firmly established, we see no radical formation in either the oxidative or reductive directions. This behavior is typical of flavoproteins. The flavoprotein oxidases, when reduced with dithionite, stabilize nearly quantitative amounts of anion radical, whereas the methylviologen reduction approaches a system of thermodynamic reversibility (Stankovich et al., 1978). The inability of butyryl-CoA dehydrogenase to thermodynamically stabilize EFlH$^\bullet$ is consistent with the preliminary titrations done by Engel and Massey (1971a), where little or no EFlH$^\bullet$ is stabilized. Williamson has also seen the formation of a nonquantitative amount of EFlH$^\bullet$ upon reduction with dithionite.[3] For these reasons, the EFlH$^\bullet$ may be kinetically stabilized in nonquantitative amounts but is not thermodynamically stable. Without the presence of thermodynamically stabilized EFlH$^\bullet$, it is therefore only possible to calculate an E_m for this electron transfer and to say that the second electron's potential is more positive than the first electron's potential by at least 180 mV (Clark, 1960).

The behavior of BCD at pH 5.5, 6.0, and 7.0 shows a reversible well-behaved 2e$^-$, 1H$^+$ transfer. From these data,

$$EFl_{ox} + 2e^- + 1H^+ \rightleftarrows EFl_{red}H^-$$
$$E_{m7} = -0.079 \text{ V vs. SHE}$$

binding constants have been determined, showing that the reduced FAD is more tightly bound to apo-BCD than oxidized FAD by a factor of 4.95×10^{-5} at pH 7.0 and by a factor of 1.73×10^{-4} at pH 6.0.

At pH values more basic than 7.0, the redox experiments clearly show the E_m of the BCD are much more negative than those projected for the active enzyme. If the pH dependence of BCD continues to be 30 mV/pH unit, an E_m value at pH 7.8 is predicted to be −0.102 V vs. SHE. Even if the oxidized form of flavin or some group near the active site deprotonated above pH 7, thus causing a two-proton, two-electron transfer, the expected potential at pH 7.8 would be −0.125 V. Therefore, the observed enzyme midpoint potential at pH 7.8 of −0.200 V lies far more negative than any predicted value. A similar comparison can be made at pH 8.5. At both pH values, the potential of BCD approaches that of free FAD ($E_{m7.8}$ = −0.235 V, and $E_{m8.5}$ = −0.255 V). As the BCD loses activity, it is probable that some of the bonds or interactions that are crucial to enzyme activity are broken, causing the E_m values for BCD to become similar to those of FAD.

The instability of BCD at pH values above 7.0 was very surprising; all previous work had been done at pH 7.0. There was no previous report of the stability of this enzyme at other pH values. Indeed, on the basis of our work, the enzyme should be purified at pH 6.0, since it is most stable at this pH. However, the "green" form of BCD which is bound to CoASS$^-$ may be more stable than the yellow CoASH-free form of the BCD studied here. The green form is present up through the final stages of purification.

Because little work has been done on this class of enzymes, the comparison of BCD to other dehydrogenases is difficult. We may, however, make some comparisons to *Paracoccus denitrificans* glutaryl-CoA dehydrogenase (which may serve as a model for the mitochondrial system) and general acyl-CoA dehydrogenase from the mammalian system. Glutaryl-CoA dehydrogenase is a tetramer with one FAD molecule per subunit like BCD. Both dithionite and EDTA–light reduction fail to show formation of the radical species but rather proceed directly to the 2e$^-$-reduced dehydroflavin (Husain & Steenkamp, 1985). GCD showed maximal stability at pH 5.0 and lost activity readily at pH values above 7.0, although this enzyme had a pH optimum of 8.0–8.5 (Husain & Steenkamp, 1985). BCD has a pH optimum at 8.02 (Williamson & Engel, 1984), yet shows highly irregular behavior above pH 7.0, like GCD.

General acyl-CoA dehydrogenase shows a larger amount of blue radical stabilized during dithionite titration; 70% is estimated from the molar absorptivity and the figure in Thorpe et al. (1979). This is much greater than the maximal 19% one sees with BCD and none for GCD. This general acyl-CoA dehydrogenase radical (20%) is also stabilized during potentiometric experiments (Gustafson et al., 1986), which is in contrast to the behavior described for BCD in this work. The potential of general acyl-CoA dehydrogenase from the mammalian system is reported to be −0.120 V vs. SHE at pH 7.1 (Gustafson et al., 1986), which differs from the potential of the bacterial system reported here by 40 mV. In work done by Gorlick et al. (1985), one sees changes in the properties of general acyl-CoA dehydrogenase upon substrate binding, with the radical species changed from blue neutral to red anion radical. Also, the potential of electron transfer appears to be shifted closer to ETF upon binding of substrate.

Although comparison is difficult because this class of enzymes is just beginning to be studied, we do see some simi-

larities. All three of these enzymes form blue radical upon reduction by artificial means. BCD appears to be more similar to the GCD in its general behavior because neither thermodynamically stabilizes the EFlH$^{\bullet}$ and the enzymes appear to be most stable in the pH range of 5.0–7.0.

The mechanism of electron transfer for the bacterial fatty acid system is described in Scheme I (Williamson, 1983). The pathway of electron transfer is the reverse of the above path for the mammalian system. The experimentally determined potentials are consistent with the direction of electron flow, with the exception of the substrate couple whose "conditional redox potential" is 40 mV more negative than the E_{m7} of BCD (Gustafson et al., 1986). This difference would argue for regulation of BCD's potential upon substrate binding. We have shown that the E_m of BCD is shifted negative by 100 mV upon binding of the substrate analogue acetoacetyl-CoA. The more negative potential of the substrate-bound enzyme makes electron transfer to the substrate more thermodynamically feasible. Regulation by substrate appears to occur with general acyl-CoA dehydrogenase and has been clearly demonstrated with D-amino-acid oxidase from pig kidney in this laboratory (Van den Berghe-Snorek & Stankovich, 1985).

The mammalian and the bacterial enzymes that catalyze fatty acid metabolism may differ significantly in their mechanism of regulation of redox properties since electron transfer occurs in opposite directions in the two types of organisms. From the limited data available, it appears that the two bacterial enzymes BCD and GCD are more similar to one another than to the mammalian general acyl-CoA dehydrogenase. Although the redox potentials of the free general acyl-CoA dehydrogenase do not differ markedly from that of BCD reported here, there is evidence that substrate binding causes the potential of the mammalian enzyme to shift in a positive direction toward the potential of the electron acceptor for the enzyme ETF (E_m = −0.025 V). However, in the bacterial system, ETF is the electron donor to BCD, and preliminary results indicate that the potential of the bacterial ETF is more negative than that of BCD. Our evidence indicates that bacterial enzyme is regulated by substrate; its potential is shifted in the negative direction, making the transfer of electrons to substrate more probable. These differences in the redox processes in metabolism are interesting in themselves and deserve more study; they could perhaps be exploited in the design of antibiotics, e.g., finding an antibiotic with the proper potential to block fatty acid metabolism in bacteria without affecting that of the host organism.

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