Biochemistry **1987**, *26*, 2627–2632 2627

addition, since our window for observation is severely limited, carbonyl absorptions with greater frequency shifts than that which we have observed could be present in some of these complexes. The possibility also exists that the chemical exchange process observed in NMR experiments is not one between states differing in carbonyl polarization and thus does not substantially effect the IR frequency.

Summary. Significant polarization of the carbonyl of OAA occurs upon binding to the active site of citrate synthase. This is the first observation of substrate destablization by both NMR and IR.

ACKNOWLEDGMENTS

We thank the McDonnell Center for Space Sciences and the Department of Physics, Washington University, for use of the FTIR spectrometer.

REFERENCES

Barrow, G. M. (1953) *J. Chem. Phys. 21*, 2008.

Bayer, E., Bauer, B., & Eggerer, H. (1981) *Eur. J. Biochem. 120*, 155.

Belasco, J. G., & Knowles, J. R. (1980) *Biochemistry 19*, 472.

Belasco, J. G., & Knowles, J. R. (1983) *Biochemistry 22*, 122.

Bellamy, L. J. (1968) *Advances in Infrared Group Frequencies*, p 122, Chapman & Hall, London.

Bellamy, L. J., & Williams, R. L. (1957) *J. Chem. Soc.*, 4294.

Clemett, C. J. (1970) *J. Chem. Soc. D*, 211.

Eggerer, H. (1965) *Biochem. Z. 343*, 111.

Eggerer, H., & Remberger, U. (1963) *Biochem. Z. 337*, 202.

Fisher, J., Belasco, J. G., Khosla, S., & Knowles, J. (1980) *Biochemistry 19*, 2895.

Johansson, C.-J., Mahlen, A., & Pattersson, G. (1973) *Biochim. Biophys. Acta 309*, 466.

Kokesh, F. C. (1976) *J. Org. Chem. 41*, 3593.

Kurz, L. C., Ackerman, J. J. H., & Drysdale, G. R. (1985) *Biochemistry 24*, 452.

Malthouse, J. P. G. (1986) *Prog. Nucl. Magn. Reson. NMR Spectrosc. 18*, 1.

Remington, S., Wiegand, G., & Huber, R. (1982) *J. Mol. Biol. 158*, 111.

Srere, P. A. (1966) *J. Biol. Chem. 241*, 2157.

Srere, P. A. (1969) *Methods Enzymol. 14*, 3.

Tiffon, B., & Dubois, J.-E. (1978) *Org. Magn. Reson. 11*, 295.

Tsubomura, H. (1956) *J. Chem. Soc. Jpn. 77*, 962.

Weidman, S. W., & Drysdale, G. R. (1979) *Biochem. J. 177*, 169.

Wiegand, G., & Remington, S. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 97.

Wiegand, G., Remington, S., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 174*, 205.

Regulation of the Butyryl-CoA Dehydrogenase by Substrate and Product Binding[†]

Marian T. Stankovich* and Sean Soltysik
Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455

Received September 10, 1986; Revised Manuscript Received December 15, 1986

ABSTRACT: Until now, workers in the field of fatty acid metabolism have suggested that the substrates are isopotential with the enzymes and that the reactions are forced to completion by the formation of charge-transfer complexes [Gustafson, W. G., Feinberg, B. A., & McFarland, J. T. (1986) *J. Biol. Chem. 261*, 7733–7741]. To date, no experimental evidence for this hypothesis exists. The work presented here shows that the butyryl-CoA/crotonyl-CoA couple is not isopotential with the enzymes with which it interacts. The potential of the butyryl-CoA/crotonyl-CoA couple ($E^{\circ\prime} = -0.013$ V) is significantly more positive than the potential of either of the enzymes with which it interacts, bacterial butyryl-CoA dehydrogenase ($E^{\circ\prime} = -0.079$ V) and mammalian general acyl-CoA dehydrogenase ($E^{\circ\prime} = -0.133$ V). These data imply that the regulation of enzyme potential is essential for any electron transfer from substrate to enzyme to occur in mammalian or bacterial systems. In support of this assertion, a significant shift in potential for bacterial butyryl-CoA dehydrogenase (an analogue of the mammalian enzyme) in the presence of butyryl-CoA and crotonyl-CoA is reported. The potential is shifted positive by 60 mV. Larger potential shifts will undoubtedly be observed with the mammalian enzyme, which would be consistent with the catalytic direction of electron transfer.

We were interested in studying the interactions of the fatty acyl-CoA's[1] with their dehydrogenases in bacterial and mammalian systems. From extensive work done on GACD, we know that this enzyme binds a wide variety of substrates but is reduced to different degrees by different substrates (Thorpe et al., 1979). The better substrates reduce the enzyme, and their products also form charge-transfer bands with the reduced enzyme. Poorer substrates partially reduce GACD. Binding is thought to play an important role in the extent of reduction. Thorpe et al. (1979) have found that with optimal substrate the degree of enzyme reduction is controlled by the relative tightness of binding of the substrate and product to the oxidized and reduced enzyme, respectively. This implies that, for titrations with optimal substrate, the product binds tighter to the reduced enzyme, favoring reduction of FAD,

---

[†] This work was supported by a grant from the National Institutes of Health (GM29344).

* Author to whom correspondence should be addressed.

[1] Abbreviations: CoA, coenzyme A; BCD, butyryl-CoA dehydrogenase; GACD, general acyl-CoA dehydrogenase; 8ClRF, 8-chlororiboflavin; PYC, pyocyanine; ETF, electron-transfer flavoprotein; CCoA, crotonyl-CoA; BCoA, butyryl-CoA; $E^{\circ\prime}$, midpoint potential; IDS, indigodisulfonate; ITRS, indigotrisulfonate; FACoA, *trans*-β-2-furylacryloyl-CoA; FPCoA, β-2-furylpropionyl-CoA; MV, methylviologen; $\hat{E}^{\circ\prime}$, conditional equilibrium potential; SHE, standard hydrogen electrode. Anything with subscript ox or red refers to the oxidized or reduced form of that species.

0006-2960/87/0426-2627$01.50/0 © 1987 American Chemical Society

while nonoptimal substrates bind the oxidized enzyme tighter. Binding must play an important role since it has been postulated that the potentials of the individual fatty acyl-CoA's should not differ appreciably (Gustafson et al., 1986; Thorpe et al., 1979). In support of the importance of product binding to the reduced GACD, Gorelick et al. (1985) showed that the potential of GACD bound to product shifted to a value near that of mammalian ETF (−0.023 V). Despite these observations, no measurement of redox potential shifts of GACD in the presence of substrate has been reported, and only approximate values for the binding constants have been reported (Ghisla et al., 1984; McKean et al., 1979).

We were confident that the same regulation that was seen in mammalian GACD would be operating in bacterial BCD and that redox studies would show this regulation. We have recently measured the potential of free BCD to be −0.079 V vs. SHE (Fink et al., 1986). The potential of the BCoA/CCoA couple is more uncertain [−0.015 V (Hauge, 1956), +0.18 V (Green et al., 1954), and −0.126 V (Gustafson et al., 1986)]. The most recently determined value is 50 mV more negative than that for BCD, requiring some regulation if CCoA is to be the electron acceptor for the much more positive BCD. In order to clarify the effects of regulation, we redetermined the potential of the substrate couple and studied the effects of binding on the BCD potentials.

MATERIALS AND METHODS

Butyryl-CoA dehydrogenase was prepared from *Megasphaera elsdenii* as previously reported (Fink et al., 1986; Williamson & Engel, 1984; Engel & Massey, 1971). The dye pyocyanine was photochemically synthesized by the method of McIlwain (1937) from phenazine methosulfate purchased from Aldrich. Indigotrisulfonate was purchased from Sigma at 75% purity, and indigodisulfonate was the gift of Dr. Fred Guengerich. 8-Chlororiboflavin was the gift of Dr. J. P. Lambooy, University of Maryland. Butyryl-CoA and crotonyl-CoA were obtained from Sigma or Pharmacia P-L Biochemicals and were dissolved in water and stored frozen. They are stable for several months in this state.

The spectroelectrochemical cell methodology was as previously described (Stankovich, 1980; Stankovich & Fox, 1983). Electrochemical experiments were performed with a PAR 173 potentiostat and a PAR 175 coulometer. Apple 2+ or 2E interfaced Cary 210 and 219 spectrophotometers with thermostated cell compartments were used.

All experiments were done in 0.1 M KP_i buffer at pH 7.0 and 15 °C. IDS and PYC were titrated in the presence of 100 μM BCoA and 100 μM CCoA. The midpoint potentials and spectra were identical with those of the titrations performed in buffer. ITRS was titrated in buffer alone.

Experiments to determine the midpoint potential of the BCoA/CCoA couple were performed as follows. Several ratios of BCoA to CCoA, yielding a total CoA concentration of 300 μM, were added to the spectroelectrochemical cell with 100 μM MV and 20 μM PYC. ITRS (10 μM) was also added to the experiments with BCoA/CCoA ratios of 10:1, 5:1, and 1:1. Buffer was added to give a total volume of 4.0 mL. The side arm contained 0.02 mL of stock BCD at a concentration of 1.8×10^{-4} M. The entire contents of the cell were degassed, and the system was electrochemically reduced to a point on either the positive or negative side of the expected equilibrium position. Electrochemical reduction was performed to remove residual oxygen and to require less turnover of substrate and product to achieve equilibrium. Too much turnover would significantly alter the ratio of BCoA to CCoA. The BCD in the side arm was added in at this point, yielding a final concentration of 1 μM, and the system was allowed to equilibrate.

Two experiments similar to those performed by Hauge (1956) were done. One started with BCoA and PYC_{ox}, and the other started with CCoA and PYC_{red}. The cell cuvette contained 1 μM BCD, 26 μM PYC, and 100 μM MV, while the side arm held 0.1 mL of 2 mM BCoA or CCoA. For the experiment with BCoA, the system was reduced electrochemically to remove all the oxygen from the cell, and then the BCoA was added from the side arm to give a final concentration of 50 μM. For the experiment with CCoA, the PYC was reduced completely and then the CCoA was added.

The hydratase control was done under aerobic and anaerobic conditions. Under aerobic conditions, 1 μM BCD and 100 μM CCoA were mixed in a cell, and the decrease in absorbance at 280 nM ($\Delta\epsilon = 3.6$ mM^{-1} cm^{-1}) was monitored. This decrease corresponds to the conversion of *trans*-2-enoyl-CoA thio esters to their 3-OH derivatives (Steinman & Hill, 1975). For the anaerobic experiment, 200 μM BCoA, 20 μM CCoA, 12.5 μM MV, and buffer were degassed in the cell. Charge was added to remove oxygen, and 0.02 mL of BCD was added from the side arm. As before, the A_{280} was monitored.

Experiments to determine the midpoint potential of BCD in the presence of BCoA and CCoA were performed under conditions similar to those used to determine the midpoint potential of BCoA/CCoA. The main difference was that the amount of BCD used was increased from 1 to 15 μM. For these experiments, the cuvette contained BCD, 100 μM MV, 20 μM PYC, and buffer. Upon addition of BCoA and CCoA from the side arm, the total CoA concentration was 300 μM. Ratios of 2:1 and 1:1 BCoA to CCoA were used for these experiments. The BCD and PYC were reduced electrochemically to a point near the expected equilibrium position. The BCoA and CCoA were added, and the system was allowed to equilibrate. As described previously, experiments utilizing 10:1 BCoA to CCoA ratios also contained ITRS.

The titration of BCD by BCoA was accomplished by degassing 14.2 μM BCD, 100 μM MV, and buffer in the cuvette of a spectroelectrochemical cell. The system was then reduced electrochemically to remove any oxygen. Aliquots of anaerobic 2 mM BCoA were added from a gas-tight syringe into the cell.

RESULTS

Potentiometric Measurements of the BCoA/CCoA Couple. From a series of preliminary experiments described below, we arrived at optimal conditions for more precise experiments. In a recent publication of Gustafson et al. (1986), the potential of the BCoA/CCoA couple is given as −0.126 V. Assuming this value to be correct, a 10:1 ratio of BCoA/CCoA would give a potential 0.030 V negative of this or 0.156 V. A good redox indicator at this potential is 8ClRF (−0.150 V). In designing a preliminary experiment to measure the $E^{\circ\prime}$ of the BCoA/CCoA couple, we anaerobically added a 10:1 mixture of BCoA/CCoA from the side arm into a solution that was 10 μM each in 8ClRF and BCD. To our surprise, the measured potential of the system at equilibrium was quite positive (−0.070 V). Since the potential was 0.080 V positive of the $E^{\circ\prime}$ for 8ClRF, the potential was not well poised. The equilibrium spectrum was hard to interpret since the spectrum of 8ClRF is similar to that of BCD, but it appeared that the total flavin was $^2/_3$ oxidized at equilibrium. However, at times longer than 5 h, we did see the development of a large amount of charge-transfer band located at 580 nm, which indicated that BCD was bound to acetoacetyl-CoA, the final product of the hydratase reaction (Engel & Massey, 1971b).

We then performed similar experiments with IDS (−0.085 V) as the redox indicator. In these experiments, a 10:1 ratio of BCoA to CCoA was tipped anaerobically into a solution of electrochemically reduced IDS and BCD (10 μM each). At equilibrium, the IDS was 95% oxidized and the BCD was half-reduced (on the basis of molar absorptivities of the electrochemically reduced enzyme). The potential indicated by both the electrode reading and the spectral properties of IDS was −0.043 V. Equilibrium was fairly well established after 1 h, and the spectra were stable for another hour. At longer times, 6 h to overnight, the spectra changed slowly and the potential drifted negative. When oxygen was admitted to the cell, the spectrum of the oxidized flavin showed that the flavin peak had shifted, indicating some binding of acetoacetyl-CoA.

These preliminary experiments yielded the following information. First, the potential of the BCD bound to substrate and/or product is significantly more positive than that of free BCD, with an $E^{\circ\prime}$ of approximately −0.043 V. Second, the BCoA/CCoA couple has a potential similar to that of substrate-bound enzyme (−0.013 V). Third, we confirmed that BCD has intrinsic hydratase activity (Ellison & Engel, 1986; Lau et al., 1986; Engel & Massey, 1971b). Initially, CCoA is hydrated to form β-hydroxy-BCoA, which is in turn oxidized to yield acetoacetyl-CoA (Engel & Massey, 1971b). We know that acetoacetyl-CoA binds tightly to BCD (Engel et al., 1984), shifting its potential negative to −0.18 V and causing significant spectral changes (charge-transfer band at 580 nm and a shift of the 450-nm peak to 432 nm) (Fink et al., 1986). This could have been a serious problem with this system, since we did not know how well the substrate or product would compete with the hydratase products for the active site nor how fast the hydratase activity depletes the concentration of CCoA. Therefore, in more careful experiments, we used less enzyme (1 μM) to lower the hydratase activity, and we tried to arrange starting conditions so that equilibrium could be established more quickly.

In order for the electrode potential to be well poised in the presence of relatively high concentrations of BCoA and CCoA, a high concentration of redox indicators is necessary (10–20 μM). It is also important for the total CoA concentration to be below 500 μM, as the electrode potential does not stabilize well at 500 μM CoA concentration.

It appeared that PYC ($E^{\circ\prime}$ = −0.013 V) would be a good redox indicator for 1:1 and lower ratios of BCoA to CCoA. In these experiments, PYC was taken to half-reduction, and BCD was added from the side arm. The system equilibrated in 1 h, and the potential and spectra remained stable for the next 2 h. After equilibration, the equilibrium distribution and potential of the dye were perturbed electrochemically by adding 3 mC of oxidative charge. Then PYC spectrum and potential always recovered their previous equilibrium position quickly (5–10 min), indicating that they were communicating through the enzyme with the vast bulk of substrate and product that buffered the potential of the system. The 3 mC of reductive charge was added to the system, and the potential and spectrum of PYC recovered as quickly as before. These potentials were stable for an additional 3–4 h and then drifted negative by 0.010 V overnight. The equilibrium potentials for these BCoA to CCoA ratios are shown in the Nernst plot (Figure 1).

In order to obtain the more reduced points for the Nernst plot, we needed a dye with a more negative potential than PYC but more positive than IDS. We tried ITRS since its potential (−0.057 V) is ideal. Unfortunately, ITRS did not equilibrate well with BCD. As in earlier experiments, after equilibrium had been achieved, we perturbed the equilibrium by adding 3 mC of reductive charge, but the spectrum and the potential did not recover to the same equilibrium point even after 2 h. We concluded that ITRS was too negatively charged to get into the active site of BCD, so we tried a mixture of PYC, which is neutral or positively charged in its redox states, and ITRS. This worked well with 5:1 and 10:1 mixtures of BCoA/CCoA. We calculated the potentials of both dyes from their respective spectra at equilibrium, and they were both within 0.005 V of the electrochemically measured potential, indicating that the system was at equilibrium. When perturbed by the addition of oxidative or reductive charge, the system recovered to its equilibrium position within 15 min. These points completed the Nernst plot shown in Figure 1. The $E^{\circ\prime}$ value is −0.013 V and the slope is 0.030 V, consistent with a reversible two-electron transfer.

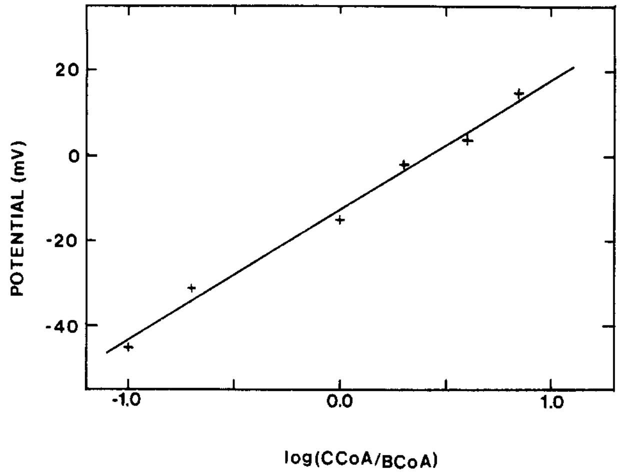


FIGURE 1: Nernst plot of equilibrium potentials from experiments with 10:1, 5:1, 1:1, 1:2, 1:4, and 1:7 ratios of BCoA to CCoA. The total CoA concentration is 300 μM. All experiments contain 1 μM BCD, 20 μM PYC, and 100 μM MV in 0.1 M KP$_i$, pH 7.0, at 15 °C. Experiments with 10:1 and 5:1 ratios of BCoA to CCoA also have 10 μM ITRS.

Two experiments patterned after Hauge (1956) were done to ensure that our potential for the BCoA/CCoA couple was correct by approaching equilibrium from two directions. By starting with PYC$_{\mathrm{ox}}$ in one case and PYC$_{\mathrm{red}}$ in the other, we were able to set definite limits on the $E^{\circ\prime}$. We had previously found the $E^{\circ\prime}$ for PYC to be −0.013 V in 0.1 M KP$_i$, pH 7.0, at 15 °C. When PYC$_{\mathrm{ox}}$ was mixed with BCoA in the presence of catalytic BCD, about half of the PYC was reduced, and after 8 h the spectral changes were very slight. By calculating an equilibrium constant ($K = [\mathrm{PYC_{red}}][\mathrm{CCoA}]/[\mathrm{PYC_{ox}}][\mathrm{BCoA}]$) at this point, we obtained a potential difference of 0.010 V with the BCoA/CCoA couple more positive, or −0.003 V. When PYC$_{\mathrm{red}}$ is mixed with CCoA, the potential difference calculated at equilibrium is 0.020 V with the BCoA/CCoA couple more negative, or −0.033 V. At equilibrium, there were significant amounts of all four species, and thus an accurate equilibrium constant could be calculated. Therefore, it can be stated with certainty that the $E^{\circ\prime}$ for the BCoA/CCoA couple is between −0.003 and −0.033 V. This is in good agreement with our value of −0.013 V, as calculated in previous experiments. The long times may account for the wide range between the two calculated midpoints since there may have been significant hydratase activity and oxygen leakage.

We said previously that BCD has intrinsic hydratase activity. We also implied that, at short times and at low enzyme concentrations, this is not a problem. This assertion is based on the following experiment in which hydratase activity was measured. The rate of conversion of CCoA was 2 μM/h under aerobic conditions. The hydratase activity was again measured

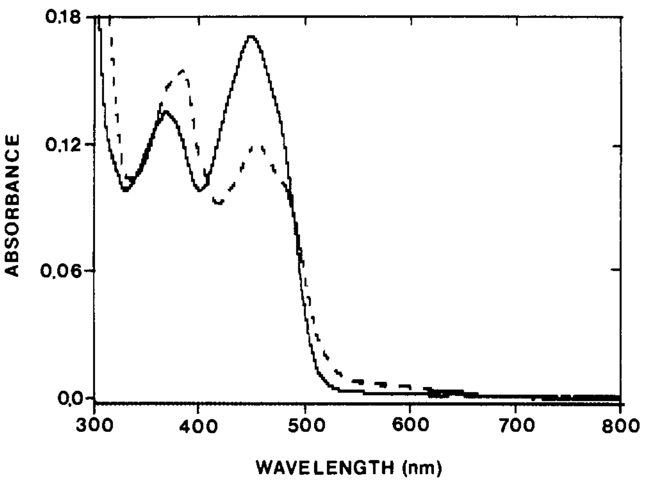


FIGURE 2: Spectrum of 12.5 μM BCD at measured potential of −0.015 V vs. SHE before (—) and after (−−) the addition of substrate and product. Initially, a solution containing BCD, 20 μM PYC, and 100 μM MV in 0.1 M KP_i, pH 7.0, at 15 °C was electrochemically reduced to a potential of −0.015 V where PYC was half-reduced. The PYC spectrum has been subtracted for clarity. A mixture of BCoA/CoA was then added anaerobically from the side arm to give a final concentration of 150 μM each. The spectrum shown is the equilibrium spectrum corrected for pyocyanine.

under anaerobic conditions, and the rate was 1.3 μM/h. In either case, for 1–2 h of equilibration time, low enzyme concentration, and high substrate and product concentrations, the hydratase activity is negligible.

$\hat{E}^{\circ\prime}$ of BCD in the Presence of BCoA and CCoA. From our preliminary experiments, we knew that the $E^{\circ\prime}$ of BCD shifted positive when BCoA (200 μM) and CCoA (20 μM) were present. We therefore performed experiments with 10–15 μM enzyme so we could monitor its spectral changes as well as the equilibrium potential. For these experiments, BCD was in the cuvette with the dye, and BCoA and CCoA were tipped in from the side arm. The potentials were established quickly (less than 1 h) and were within 0.003 V of the values previously established for the same BCoA to CoA ratios with catalytic amounts of BCD. Three experiments were done with BCoA to CCoA ratios of 1:1, 2:1, and 10:1. Figure 2 shows the results of the 1:1 experiment. When the equilibrium spectrum of this experiment was corrected for PYC, the spectrum for the enzyme alone showed a long-wavelength absorbance characteristic of a charge-transfer band between reduced enzyme and CCoA. The A_{450} was 56% of that obtained in an electrochemical reduction of free enzyme. The high absorbance in the 380-nm region may be due to anion radical. This phenomenon will be investigated in future work.

One hour after equilibrium was established, oxygen was added to the cell. BCD reoxidized over a 5–10-min period, yielding a spectrum similar in magnitude to the starting spectrum. The spectrum was slightly different from the starting spectrum since the oxidized BCD binds to substrate. There was no shift in the 450-nm band to 432 nm, which would indicate the presence of significant hydratase activity. Since the measured equilibrium potential was the same as that calculated in the experiment using catalytic BCD and a 1:1 ratio of BCoA to CCoA, we conclude that we had no significant amount of acetoacetyl-CoA produced at the higher enzyme concentrations. The facile reaction with oxygen indicates that the bacterial BCD has an accessible active site even when bound to substrate and product.

In order to calculate the concentrations of oxidized and reduced BCD present at equilibrium, we used the following molar absorptivities. The free oxidized BCD and CCoA-bound oxidized BCD have similar ϵ_{450} [ϵ_{450}(free BCD) = 14 400 M^{-1} cm^{-1} (Williamson & Engel, 1984); ϵ_{454}(CCoA-bound BCD) = 13 900 M^{-1} cm^{-1} (Engel & Massey, 1971b)]. Both BCoA and CCoA bind tightly to the reduced form of GACD (L. Schopfer and V. Massey, personal communication). We assumed similar tight binding for BCD, and thus no free reduced BCD exists. The molar absorptivities used for BCoA-bound reduced enzyme (ϵ_{450} = 2000 M^{-1} cm^{-1}) and for CCoA-bound reduced enzyme (ϵ_{450} = 4000 M^{-1} cm^{-1} and ϵ_{580} = 3000 M^{-1} cm^{-1}) were obtained from work on mammalian GACD where quantitative amounts of the charge-transfer complex and quantitative amounts of BCoA-bound reduced enzyme were obtained in stopped-flow experiments (L. Schopfer and V. Massey, personal communication). Using these molar absorptivities, we calculated the concentration of total oxidized BCD (7.7 μM) and both forms of reduced species for the 1:1 experiment (3.1 μM, BCoA bound; 1.7 μM, CCoA bound). Using these values in the Nernst equation and the measured potential (−0.012 V), we calculated a $\hat{E}^{\circ\prime}$ for BCD of −0.018 V vs. SHE. A similar $\hat{E}^{\circ\prime}$ value was obtained at a BCoA to CCoA ratio of 2:1 ($\hat{E}^{\circ\prime}$ = −0.020 V). These two experiments clearly show that the BCD potential is shifted positive by 60 mV in the presence of this concentration of substrate and product. This shift indicates that the ratios of binding constants for reduced and oxidized BCD differ by a factor of 100, with the reduced form being more tightly bound.

Experiments with a 10:1 ratio of BCoA to CCoA gave an $\hat{E}^{\circ\prime}$ value of −0.034 V. Because of difficulties in correcting for the BCD spectrum at 580 nm due to ITRS, no information on the amount of the charge-transfer band was obtained.

These experiments clearly show that, in the presence of the substrate and product, the potential of the enzyme shifts to a value where BCoA reduces the enzyme.

Titration of BCD with BCoA. Figure 3 shows the progress of the titration of BCD with BCoA. The plot of A_{450} vs. amount of BCoA added is not linear, suggesting that the reduction is not quantitative and that an equilibrium exists between all four species (oxidized and reduced BCD and oxidized and reduced substrate) throughout the entire titration. The percent reduction compared to electrochemical reduction is 75%. If we calculate the concentration of reduced and oxidized BCD species throughout the titration, we can use these to calculate a conditional potential difference ($\Delta\hat{E}^{\circ\prime}$) for the enzyme and substrate couples. On the average, we obtain $\Delta\hat{E}^{\circ\prime}$ = 15 mV with the $\hat{E}^{\circ\prime}$ for BCD being the more positive. We already know that the enzyme $E^{\circ\prime}$ shifts in the presence

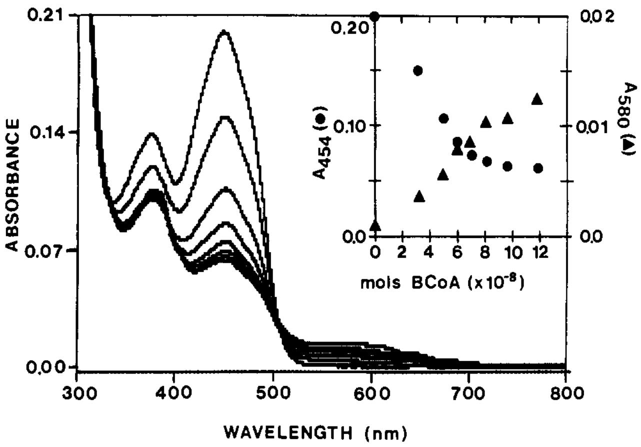
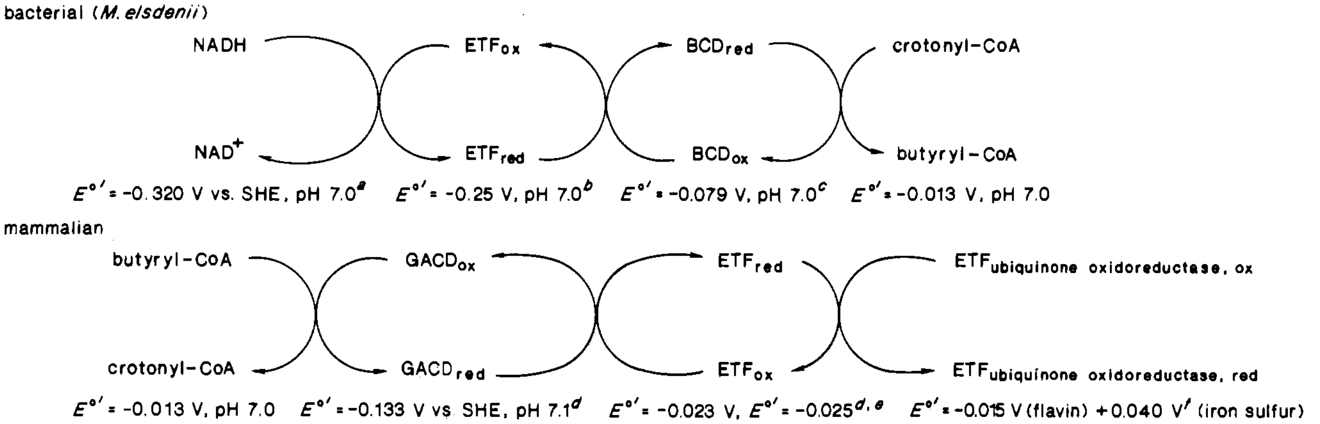


FIGURE 3: Anaerobic titration of 13.9 μM BCD in 0.1 M KP_i, pH 7.0, at 15 °C by aliquots of 2 × 10^{-3} M BCoA. Initial volume = 3.7 mL. All spectra are corrected for dilution. The inset is a plot of A_{454} (●) vs. moles of BCoA added and A_{580} (▲) vs. moles of BCoA added.

Scheme I



[a] Underwood & Burnett, 1973. [b] C. Pace, unpublished results. [c] Fink et al., 1986. [d] Gustafson et al., 1986. [e] Husain et al., 1984. [f] Ruzicka & Beinert, 1977.

of substrate and product ($\hat{E}^{\circ\prime} = -0.019$ V) to a value close to the $E^{\circ\prime}$ of free substrate and product (−0.013 V). This experiment shows that the binding by the enzyme causes the potential of the substrate to change also.

DISCUSSION

Because of the difficulty in monitoring spectrally the changes in concentration of the BCoA/CCoA couple, we endeavored to keep these species from changing significantly during the course of the equilibrations. We have described similar values obtained for the redox potential of the BCoA/CCoA couple from a wide variety of redox indicators with different structures and different midpoint potentials. It was clearly evident that the potential of the couple was much more positive than −0.150 V (8ClRF), or −0.085 V (IDS), and even more positive than −0.057 V (ITRS).

The experiments according to the method of Hauge (1956) were exceptions to this design. The experiments were designed so that large concentration changes would occur for the substrate and product. These experiments showed reversibility most convincingly but also gave a large range for the $E^{\circ\prime}$ due to lower CoA concentrations and longer equilibration times. The potentials continued to drift throughout the experiments due to hydratase activity, and perhaps to oxygen leakage, making the final determination of equilibrium difficult. However, these experiments did set limits for the $E^{\circ\prime}$ and established the need for more closely controlled experiments.

In order to compensate for these constraints, we used a wide distribution of ratios of BCoA to CCoA (10:1 to 1:7). Also, the system was reduced close to the expected equilibrium position prior to adding in the substrate and product. However, this did not influence the final equilibrium position since the same potential was recorded (±0.003 V) regardless of the state of the system before BCoA and CCoA were added. In addition, we perturbed the equilibrium of these systems by adding oxidative and reductive charge and then observed and the rapid reestablishment of equilibrium.

The positive potential of the BCoA/CCoA couple is consistent with its role as a final electron acceptor in the bacterial system. The potential of the unbound BCD is consistent with its role as an electron donor to CCoA (see Scheme I). However, it was most interesting to see that, in the presence of BCoA/CCoA, the BCD potential shifts in the positive direction to a potential close to that of the BCoA/CCoA couple. An equilibrium was favored rather than a total electron transfer to CCoA, which would have been predicted by the free potentials. Likewise, on the basis of the potentials of the unbound enzyme, transfer of electrons from BCoA to BCD would be unfavorable. However, from the potential shifts and the titration shown in Figure 3, it is clear that BCoA can transfer electrons to the enzyme and thus establish an equilibrium.

Originally, it appeared that the positive $E^{\circ\prime}$ measured for BCoA/CCoA was totally inconsistent with BCoA acting as an electron donor to GACD ($E^{\circ\prime} = -0.133$ V; Scheme I) (Gustafson et al., 1986; Thorpe et al., 1979). We wondered how this unfavorable electron transfer could occur. Surprisingly, a solution to this dilemma is suggested by the shift in potential exhibited by BCD bound to substrate and/or product. We have seen that the BCD potential shifts positive by 0.060 V when bound to BCoA/CCoA. If the potential of GACD had to be shifted to −0.020 V, that would be a shift of 0.113 V, and the reduced form of GACD would only have to be bound tighter than the oxidized form by a factor of about 1000. In fact, evidence for this sort of potential shift has already been shown by Gorelick et al. (1984) with octenoyl-CoA. They found that when a mixture of ETF ($E^{\circ\prime} = -0.023$ V) and GACD ($E^{\circ\prime} = -0.133$ V) was titrated with dithionite, the enzymes reduced serially, as would have been expected from their respective redox potentials. However, in the presence of octenoyl-CoA, they saw that the redox potential of the GACD becomes much closer to that of ETF and reducing equivalents are shared between both enzymes. In light of what we have discovered for BCD, it is possible that the GACD potential is shifted positive, near to that of ETF.

The results presented here demonstrate clearly that binding plays a crucial role in regulation of electron transfer in fatty acid metabolism in both bacterial and mammalian systems. This is an important contribution to our understanding of fatty acid metabolism.

Our value for the redox potential of the BCoA/CCoA couple agrees with the value determined by Hauge in 1955, although the most recent $E^{\circ\prime}$ value for the BCoA/CCoA couple is given as −0.126 V by Gustafson et al. (1986). They correctly describe their value as a "conditional $E^{\circ\prime}$" since it was determined by using the potential of enzyme bound by the FACoA/FPCoA couple. Using the FACoA/FPCoA couple has the advantage that FACoA absorbs at 340 nm, and thus its concentration can be monitored spectrally during an experiment. They had previously done a transhydrogenation experiment between FACoA/FPCoA and BCoA/CCoA and discovered that FACoA/FPCoA is more negative by 0.030 V. They had also determined $E^{\circ\prime}$ for GACD as −0.133 V. By mixing 13 μM GACD with 11 μM FACoA, they determined an equilibrium constant that allowed them to calculate a potential difference of 0.023 V with the FACoA/FPCoA couple more negative, or −0.156 V. They stress that this is a conditional value since it may include energetic consequences of the preferential interaction of substrate or product with enzyme (Gustafson et al., 1986). Given this conditional value of −0.156 V for the FACoA/FPCoA couple, they then incorporated the 0.030-V difference between FACoA/FPCoA and BCoA/CCoA to arrive at a value of −0.126 V for the BCoA/CCoA couple.

It is clear that FACoA is binding to enzyme, as the authors point out in their description of spectral changes, upon addition of FACoA to GACD. This binding is likely to cause significant changes in the GACD $E^{\circ\prime}$ that they have not compensated for. In contrast, we have clearly shown that the potential for BCD is shifted 0.060 V positive when substrate and/or product are (is) bound. Considering the method by which they arrived at −0.126 V for the BCoA/CCoA couple, it is not surprising that our value is 0.113 V more positive at −0.013 V. We have calculated that binding of substrate or product to the reduced form could account for this shift.

ACKNOWLEDGMENTS

We thank Dr. J. T. McFarland and Dr. Colin Thorpe for sending us preprints of their manuscripts. We are grateful to Dr. Vincent Massey and Dr. Larry Schopfer for discussing their preliminary results.

REFERENCES

Beinert, H., & Page, E. (1957) *J. Biol. Chem. 225*, 479–497.

Ellison, P. A., & Engel, P. C. (1986) *Biochem. Soc. Trans. 14*, 158.

Engel, P. C., & Massey, V. (1971a) *Biochem. J. 125*, 879–887.

Engel, P. C., & Massey, V. (1971b) *Biochem. J. 125*, 889–902.

Engel, P. C., Williams, G., & Shaw, L. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C., & Mayhew, S. G., Eds.) pp 403–412, de Gruyter, New York.

Fink, C. W., Stankovich, M. T., & Soltysik, S. (1986) *Biochemistry 25*, 6637–6643.

Ghisla, S., Thorpe, C., & Massey, V. (1984) *Biochemistry 23*, 3154–3161.

Gorelick, R. J., Schopfer, L. M., Ballou, D. P., Massey, V., & Thorpe, C. (1985) *Biochemistry 24*, 6830–6839.

Green, D. E., Mii, S., Mahler, H. R., & Bock, R. M. (1954) *J. Biol. Chem. 206*, 1–12.

Gustafson, W. G., Feinberg, B. A., & McFarland, J. T. (1986) *J. Biol. Chem. 261*, 7733–7741.

Hauge, J. G. (1956) *J. Am. Chem. Soc. 78*, 5266–5272.

Husain, M., Stankovich, M. T., & Fox, B. G. (1984) *Biochem. J. 219*, 1043–1047.

Lau, S.-M., Powell, P., Buettner, H., Ghisla, S., & Thorpe, C. (1986) *Biochemistry 25*, 4184–4189.

McIlwain, H. (1937) *J. Chem. Soc. 2*, 1704–1711.

McKean, M. C., Frerman, F. E., & Mielke, D. M. (1979) *J. Biol. Chem. 254*, 2730–2735.

Ruzicka, F. J., & Beinert, H. (1977) *J. Biol. Chem. 252*, 8440–8445.

Stankovich, M. T. (1980) *Anal. Biochem. 109*, 295–308.

Stankovich, M. T., & Fox, B. G. (1983) *Biochemistry 22*, 4466–4472.

Steinman, H. M., & Hill, R. L. (1975) *Methods Enzymol. 35*, 136–151.

Thorpe, C., Matthews, R. G., & Williams, C. H. (1979) *Biochemistry 18*, 331–337.

Underwood, A. L., & Burnett, R. W. (1973) *Electroanal. Chem. 6*, 1–86.

Williamson, G., & Engel, P. C. (1984) *Biochem. J. 218*, 521–529.