

File ID	uvapub:1555
Filename	16336y.pdf
Version	unknown

SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type	article
Title	4-Hydroxybutyryl-CoA dehydratase from <i>Clostridium aminobutyricum</i> : characterization of FAD and iron sulfur clusters involved in an overall non- redox reaction.
Author(s)	U. Muh, I. Cinkaya, S.P.J. Albracht, W. Buckel
Faculty	FNWI: Swammerdam Institute for Life Sciences (SILS)
Year	1996

FULL BIBLIOGRAPHIC DETAILS:

<http://hdl.handle.net/11245/1.122337>

Copyright

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content licence (like Creative Commons).

4-Hydroxybutyryl-CoA Dehydratase from *Clostridium aminobutyricum*: Characterization of FAD and Iron–Sulfur Clusters Involved in an Overall Non-Redox Reaction[†]

Ute Müh,^{‡,§} Irfan Çinkaya,[‡] Simon P. J. Albracht,^{||} and Wolfgang Buckel^{*,‡}

Laboratorium für Mikrobiologie am Fachbereich Biologie der Philipps Universität Marburg, Karl-von-Frisch Strasse, D-35032 Marburg, Germany, Max-Planck-Institut für Terrestrische Mikrobiologie, Karl-von-Frisch Strasse, D-35043 Marburg, Germany, and E. C. Slater Institute, BioCentrum Amsterdam, Plantage Muidergracht 12, NL-1018 TV Amsterdam, The Netherlands

Received January 19, 1996; Revised Manuscript Received May 15, 1996[®]

ABSTRACT: 4-Hydroxybutyryl-CoA dehydratase catalyzes the reversible dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA, which involves cleavage of an unactivated β -C–H bond. The enzyme also catalyzes the apparently irreversible isomerization of vinylacetyl-CoA to crotonyl-CoA. Addition of crotonyl-CoA to the dehydratase, which contains FAD as well as non-heme iron and acid labile sulfur, led to a decrease of the flavin absorbance at 438 nm and an increase in the region from 500 to 800 nm. The protein-bound FAD was easily reduced to the semiquinone (redox equilibration within seconds) and only slowly to the hydroquinone (redox equilibration minutes to hours); the redox potentials were not unusual for flavoproteins ($E_{ox/sq} = -140 \pm 15$ mV and $E_{sq/red} = -240 \pm 15$ mV; pH 7.0, 25 °C). There was no equilibration of electrons between the flavin and the Fe-S cluster, which was difficult to reduce. After extensive photoreduction, an EPR signal indicative of a $[4Fe-4S]^+$ cluster was detected (g -values: 2.037, 1.895, 1.844). Upon exposure to air at 0 °C, the enzyme lost dehydration activity completely within 40 min, but isomerase activity dropped to about 40% of the initial value and persisted for more than a day. The properties of the protein-bound FAD are consistent with a mechanism involving transient one-electron oxidation of the substrate to activate the β -C–H bond. The putative $[4Fe-4S]^{2+}$ cluster could serve a structural role and/or as Lewis acid facilitating the leaving of the hydroxyl group.

In recent years it has become clear that the functions of Fe-S clusters are not restricted to electron transport (Emptage, 1988; Switzer, 1989; Johnson, 1994). In glutamine phosphoribosylpyrophosphate amidotransferase from *Bacillus subtilis* the available evidence suggests that a $[4Fe-4S]^{2+}$ cluster serves a purely structural role (Vollmer et al., 1983; Grandoni et al., 1989). In dihydroxy acid dehydratase it has been shown that an Fe-S cluster ($[2Fe-2S]$ in the enzyme from spinach and $[4Fe-4S]$ in the enzyme from *Escherichia coli*) activates the substrate by direct interaction (Flint & Emptage, 1988; Flint et al., 1993a). In addition, some data suggest that the cluster signals the presence of an aerobic growth environment (Flint et al., 1993b). The best studied example of a non-redox role for Fe-S centers is in the enzyme aconitase, for which it has been shown that a $[4Fe-4S]^{2+}$ cluster participates in the reaction as a Lewis acid and facilitates removal of the hydroxyl group from the substrates citrate and (2*R*,3*S*)-isocitrate to enable dehydration [for a review see Beinert and Kennedy (1989)]. Three of the iron atoms are coordinated to three cysteine residues of the enzyme, whereas the fourth iron atom interacts with the substrate (Emptage et al., 1983b; Robbins & Stout, 1989).

In the absence of substrate, aconitase is easily inactivated upon exposure to air. This is due to loss of the non-cysteine-bound iron atom, creating an EPR active $[3Fe-4S]^+$ cluster (Kent et al., 1982; Kennedy et al., 1983).

Consequently, when other oxygen sensitive hydrolyases were discovered to contain iron and acid labile sulfur, a similar role for the Fe-S clusters was proposed (Buckel, 1992; Grabowski et al., 1993) and interaction with the substrate was demonstrated in the case of bacterial L-serine dehydratase (Hofmeister et al., 1994). 4-Hydroxybutyryl-CoA dehydratase, apparently a further member of this class, has been shown to catalyze the reversible dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA (Willadsen & Buckel, 1990; Scherf & Buckel, 1993). The equilibrium constant is about 4 on the side of crotonyl-CoA. When the enzyme is incubated with vinylacetyl-CoA, the same ratio of 4-hydroxybutyryl-CoA to crotonyl-CoA is reached and equilibrium is attained at a rate equal to the reaction rate with 4-hydroxybutyryl-CoA (Scherf & Buckel, 1993). This could be interpreted as vinylacetyl-CoA being an intermediate in the reaction. Alternately, it may reflect the enzyme's ability to deprotonate at the C-2 position of a CoA-ester, which is necessary for the isomerization step. (See Scheme 1.)

The dehydratase has been purified from the strictly anaerobic organisms *Clostridium aminobutyricum* (Scherf & Buckel, 1993) and *Clostridium kluyveri* (Scherf et al., 1994) and was described as a homotetramer with a molecular mass of 56 kDa per subunit. Upon exposure to air at 25 °C, the dehydratase completely loses both activities at identical rates, with half-lives between 10 and 20 min depending on the

[†] This work was supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

[‡] Philipps Universität Marburg.

[§] Max-Planck-Institut.

^{||} E. C. Slater Institute, Amsterdam.

[®] Abstract published in *Advance ACS Abstracts*, August 15, 1996.

¹ Abbreviations: $E_{ox/sq}$, $E_{sq/red}$, and $E_{ox/red}$ are defined as the redox potentials of enzyme-bound flavin from oxidized to semiquinone, semiquinone to reduced, and oxidized to reduced, respectively; A_{438} is the absorbance at 438 nm; DMSO, dimethyl sulfoxide.

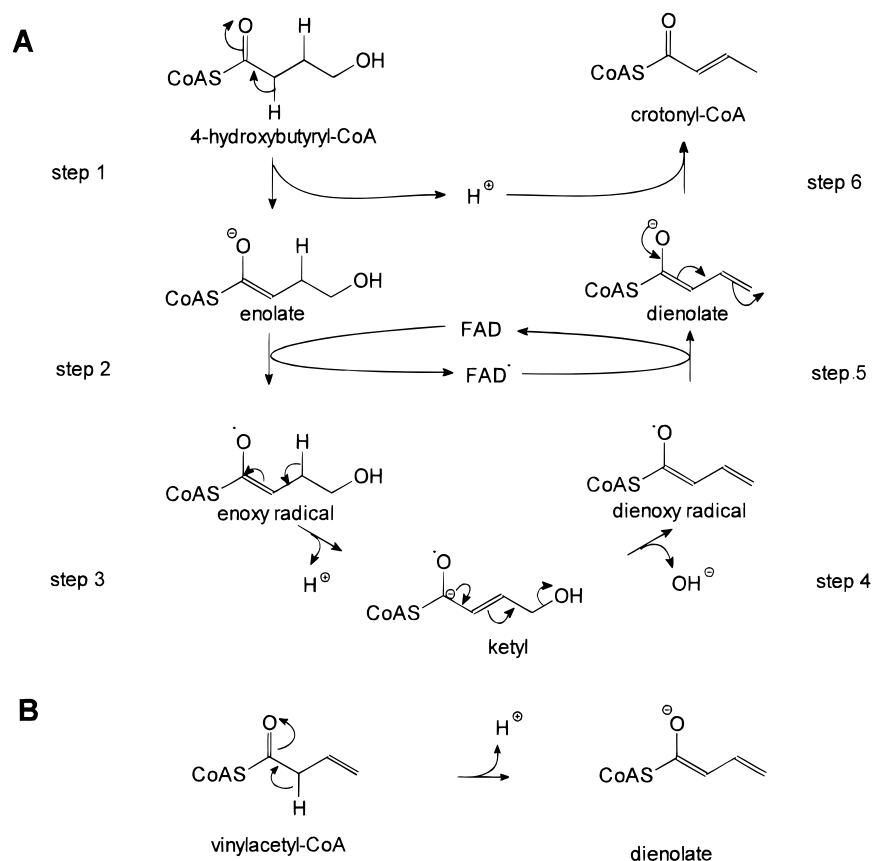
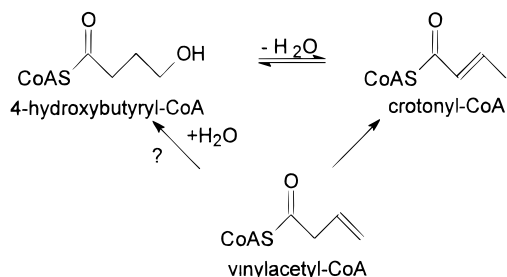


FIGURE 1: Proposed reaction mechanism for 4-hydroxybutyryl-CoA dehydratase with 4-hydroxybutyryl-CoA (A) and with vinylacetyl-CoA (B).

Scheme 1



enzyme concentration. The dehydratase contains two FAD per homotetramer as well as iron and acid labile sulfur. On the basis of the reported stoichiometry of around 16 Fe and 16 sulfur per homotetramer, it was suggested that the enzyme could contain [4Fe-4S] clusters.

The dehydration reaction must proceed by a mechanism quite different from the typical dehydration (that performed, e.g., by crotonase), since an unactivated β -H atom has to be removed. In view of the unusual reaction mechanism and the cryptic roles of the cofactors, several models have been suggested for the reaction (Scherf & Buckel, 1993; Buckel & Keese, 1995). All proposals have in common a substrate-activating role for the flavin and a polarizing function for a putative [4Fe-4S] cluster. The proposals are also based on the observation that the enzyme becomes inactive upon reduction. That is, the enzyme must be oxidized before catalysis can be initiated (Scherf & Buckel, 1993).

One quite novel hypothesis postulates the deprotonation and oxidation of the substrate to a ketyl-like anion radical as the key intermediate and the cycling of one electron onto

the flavin [Figure 1A, modified version of that proposed by Buckel and Keese (1995)]. Catalysis is initiated by abstraction of an α -proton from the substrate (step 1), followed by reduction of the flavin to the semiquinone (step 2). This yields a substrate enoxy-radical which is deprotonated at the β -carbon to a ketyl-like radical anion (step 3). It has been shown for α -hydroxy ketones that in the presence of a strong one-electron donor, a ketyl is generated which drives the elimination of the adjacent hydroxyl group (Molander & Hahn, 1986). In the model reaction the hydroxyl function is activated by tosylation; in the enzyme the Fe-S cluster as a Lewis acid would serve this purpose, leading to the dienoxy-radical species (step 4). In step 5 the flavin is reoxidized, yielding the dienolate which is finally protonated to the product crotonyl-CoA (step 6). Abstraction of a proton from C-2 of vinylacetyl-CoA would lead directly to this dienolate, which could explain the isomerase activity of the enzyme (Figure 1B).

An alternate possibility could be a sequence of hydride transfers proposed previously (Scherf & Buckel, 1993). The latter is distinct from the first proposal in that it does not involve a radical intermediate. Rather the flavin should cycle between the oxidized and the fully reduced state.

In order to investigate the mechanism by which 4-hydroxybutyryl-CoA dehydratase catalyzes its reaction, a thorough characterization of the enzyme was attempted. Spectral changes upon incubation with substrate are reported, as well as redox properties of the FAD, which support its proposed role in catalysis. EPR measurements indicate, however, that the Fe-S cluster may have a function different from that in aconitase.

MATERIALS AND METHODS

Materials. Crotonyl-CoA was prepared by the mixed anhydride method (Simon & Shemin, 1953). The concentration was determined as described for butyryl-CoA (Scherf & Buckel, 1991) but with a crotonate CoA-transferase isolated from a 5-aminovaleate fermenting organism that is able to grow on crotonate. For this, crotonyl-CoA and acetate are reacted to acetyl-CoA by incubation with crotonate CoA-transferase. In the presence of citrate synthase and oxaloacetate, acetyl-CoA is further converted to citrate and the released CoA monitored by reaction with DTNB [5,5'-dithiobis(2-nitrobenzoate)]. 4-Hydroxybutyrate CoA-transferase (Scherf & Buckel, 1991) and crotonate CoA-transferase (not published) were obtained from Dr. Uwe Scherf, Philipps-Universität Marburg. 5-Deazariboflavin was a generous gift of Dr. Vincent Massey, University of Michigan. Xanthine oxidase and crotonase were purchased from Boehringer Mannheim, Germany. 3-Hydroxyacyl-CoA dehydrogenase was from Sigma.

Instrumentation. Absorption spectra were recorded with a temperature-controlled Hewlett-Packard diode array 8452A. Rapid kinetics were measured with an SF-61MX stopped-flow apparatus (Hi-Tech Scientific, Salisbury, U.K.). EPR spectra at X-band were measured with a Bruker ESC 106 EPR spectrometer and a Varian E3 EPR spectrometer at a field modulation frequency of 100 kHz. Cooling of the sample was achieved with liquid nitrogen or with an Oxford Instruments ESR 900 cryostat regulated with an ITC4 temperature controller. Spin quantitation was calculated as described previously (Albracht, 1980). Anaerobic manipulations were performed in a glovebox (Coy Laboratories, Ann Arbor, MI) with an atmosphere of 5% H₂ in N₂. For UV/vis reduction experiments, an anaerobic glass cuvette was constructed that is equipped with two side-arms for the addition of reagents. The cuvette was made anaerobic by alternate cycles of evacuation and flushing with nitrogen which had been passed over two columns of Oxysorb (Messer Griesheim, Germany).

Cell Growth and Enzyme Purification. Cultures of *C. aminobutyricum* (DSM 2634, listed as *Clostridium* sp.) were grown anaerobically, and the enzyme was purified as described previously (Scherf & Buckel, 1993). The specific activities of the preparations varied between 2–9 units/mg of protein. Purified dehydratase was frozen rapidly in liquid nitrogen and stored anaerobically at –80 °C. Thus stored, the enzyme retained at least 80% of its activity for several months. The enzyme used in experiments reported here was judged to be at least 95% pure by Coomassie staining of polyacrylamide gels. It typically contained about 8 iron per homotetramer. The difference to the previous observation of about 16 iron per homotetramer (Scherf & Buckel, 1993), despite similar specific activities, cannot be explained.

Activity Assay. Enzyme activity was measured with a coupled assay (Scherf & Buckel, 1993) but under aerobic conditions, since this was found not to affect activity over short times. Briefly, the substrate, 4-hydroxybutyryl-CoA, was generated *in situ* by 4-hydroxybutyrate CoA-transferase from acetyl-CoA and 4-hydroxybutyrate. Reactions were started by addition of 4-hydroxybutyryl-CoA dehydratase. The product, crotonyl-CoA, was detected by monitoring the appearance of NADH after conversion to 3-hydroxybutyryl-CoA and subsequent oxidation to acetoacetyl-CoA. For this,

crotonase, 3-hydroxyacyl-CoA dehydrogenase, and NAD were included in the assay mixture. Activity was measured in 50 mM Tris-HCl, pH 9.0 at 25 °C.

Extinction Coefficient. The extinction coefficient of the enzyme was determined for each preparation by treating the enzyme with 0.2 M trichloroacetic acid for several minutes. The precipitated protein was removed by 2 min centrifugation at 15 000g, and the concentration of released FAD was measured spectroscopically in the supernatant. The enzyme concentration was then calculated based on an extinction coefficient of 11 300 M^{–1} cm^{–1} for free FAD at 445 nm (Whitby, 1953) and on the stoichiometry of 2 FAD per homotetramer. Since the number of active sites in the dehydratase is not clear, all enzyme concentrations are given for the homotetramer.

Reduction of the Enzyme. Three different methods of reduction were tested. The enzyme was photoreduced in the presence of 5-deazariboflavin as described by Massey and Hemmerich (1977, 1978). The reaction mixture contained enzyme in 50 mM potassium phosphate, pH 7.0, 1 μM 5-deazariboflavin, and either 10 mM EDTA, 20 mM oxalate, or 100 mM glycine. These different sources of reducing equivalents were used to test the susceptibility of the Fe-S cluster to the presence of chelating agents. Typically 5 μM benzyl viologen or 20 μM methyl viologen was added as redox mediator. Components were mixed in the anaerobic cuvette, except for the electron source which was pipetted into the side-arm. The cuvette was made anaerobic, the electron source was tipped in from the side-arm of the vessel and the sample irradiated with a halogen lamp (250 W). Unwanted irradiation with UV- or IR-light was prevented by placing the cuvette in a glass beaker filled with ice-water. Spectral changes were recorded after redox equilibration at 25 °C.

Reduction was also attempted by the electron-generating reaction with xanthine oxidase (Massey, 1991). Enzyme was incubated anaerobically with 0.33 mM xanthine and a redox mediator (5 μM benzyl viologen or 20 μM methyl viologen) in 50 mM potassium phosphate, at pH 7.0 and 25 °C. The reaction was started by addition of xanthine oxidase to a final concentration of about 0.02 units/mL, and the spectral changes were recorded.

A third method of reduction involved titration with dithionite and measuring the redox potential with an electrode (Ingold Pt 4805-S7/120). Typically a 5 mL sample of enzyme in the presence of 5 μM redox mediator was titrated in the glovebox with aliquots of a 5 mM dithionite solution freshly prepared in anaerobic buffer. After addition of dithionite, the enzyme mixture was incubated at room temperature until the redox electrode showed no further change in potential. A 1 mL sample was removed, spun in a microcentrifuge to remove any possible precipitate (of which there was very little), and pipetted into a cuvette. The cuvette was closed with a rubber stopper, the spectrum was measured outside the glovebox, and the sample was returned to the reaction pool.

Reaction of Reduced Dehydratase with Oxygen. Dehydratase (typically around 8 μM, 2 mg/mL) was reduced by irradiation or with the xanthine/xanthine oxidase system as described above. For this, the anaerobic cuvette was attached to a tonometer, a glass construct which minimizes oxygen contamination when loading an anaerobic solution onto the stopped-flow apparatus. The fully reduced enzyme was then

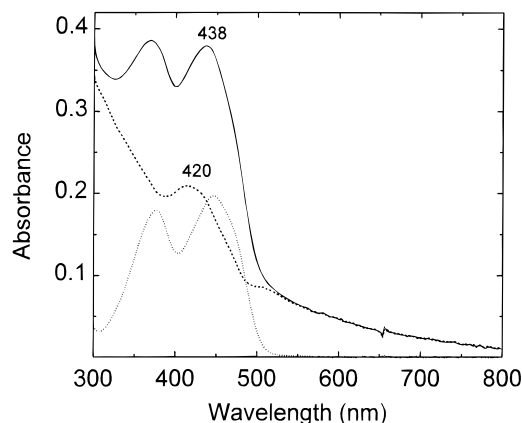


FIGURE 2: The UV/visible absorbance spectrum of oxidized dehydratase (solid line). The enzyme kept under anaerobic conditions was diluted to $8\ \mu\text{M}$ with aerobic 50 mM potassium phosphate, pH 7.0, and the spectrum was recorded within the next minute. The supernatant after treatment with 0.2 M trichloroacetic acid was corrected for dilution (dotted line). The difference spectrum (dashed line) indicates in first approximation the contribution of the Fe-S cluster to the spectrum of the holoenzyme. This approximation does not take into account any changes of the flavin absorbance due to binding.

reacted in the stopped-flow apparatus with buffer (50 mM potassium phosphate, pH 7.5) at various concentrations of oxygen at 25 °C. Buffers were oxygen equilibrated by bubbling with commercial mixtures of oxygen (100%, 50%, and 5% in nitrogen, Messer Griesheim) or by air equilibration (21% oxygen) for 15 min at 25 °C. Spectral changes were followed at 438 nm.

RESULTS

Spectral Properties. The spectrum of purified 4-hydroxybutyryl-CoA dehydratase (Figure 2) exhibited the flavin-typical absorbance at 380 nm and at 438 nm. In addition there was a broad, unresolved absorbance, stretching from >800 to 500 nm. This long-wavelength absorbance is like that caused by a slight turbidity, although the sample was routinely spun at 15 000g before spectral measurements. Also filtration through a $2\ \mu\text{m}$ filter did not affect the spectrum. After treatment with 0.2 M trichloroacetic acid, the spectrum of the supernatant was that of free FAD. The difference spectrum gives in first approximation the contribution of the Fe-S cluster to the absorbance of the intact enzyme. There are no distinct features, only a broad maximum around 420 nm. The extinction coefficient for the dehydratase was calculated to be around $48\ \text{mM}^{-1}\ \text{cm}^{-1}$ at 438 nm for the homotetramer ($24\ \text{mM}^{-1}\ \text{cm}^{-1}/\text{FAD}$). Neither oxidized nor completely reduced dehydratase in the hydroquinone state showed any significant fluorescence as observed by eye with a UV-lamp.

Inactivation by Air. It has been shown previously that incubation of the dehydratase under air at 25 °C resulted in the complete loss of the dehydratase as well as the isomerase activities at identical rates as measured by the formation of crotonyl-CoA from either 4-hydroxybutyryl-CoA or vinylacetyl-CoA, respectively. In the meantime, air inactivation has been investigated at lower temperatures. Upon incubation at 0 °C the dehydratase activity was completely lost within 40 min ($0.5\ \text{mg}$ of enzyme/mL), whereas the isomerase activity only dropped to 40% of the initial value during this time and persisted upon further incubation for

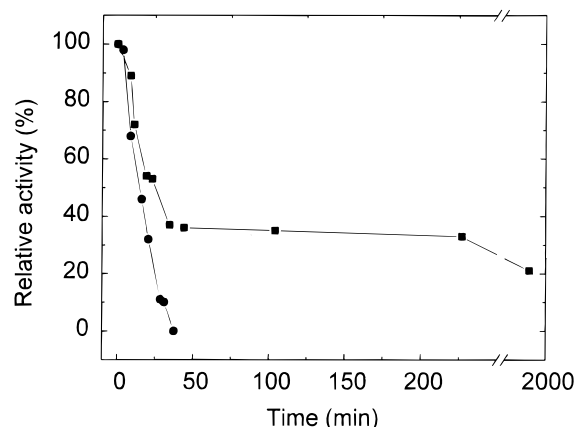


FIGURE 3: Inactivation of 4-hydroxybutyryl-CoA dehydratase with air at 0 °C: 4-Hydroxybutyryl-CoA dehydratase activity (closed circles) and vinylacetyl-CoA isomerase activity (closed squares). Enzyme was exposed to air at 0 °C and assayed as described in Materials and Methods.

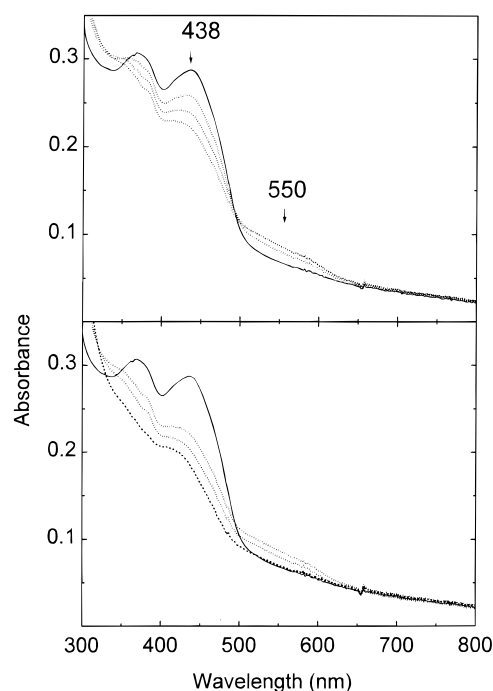


FIGURE 4: Photoreduction of the dehydratase. Top: Reduction from oxidized flavin to semiquinone. Oxidized enzyme (solid line) after irradiation with light for 8, 15, and 20 s (dotted lines); the appearance of flavin semiquinone is characterized by the increase around 550 nm. Bottom: Reduction from flavin semiquinone to flavin hydroquinone. The spectrum of the oxidized enzyme is included as reference (solid line). Further irradiation (25 and 35 s, dotted lines) led to fully reduced flavin after 125 s (dashed line). The reaction mixture contained $6\ \mu\text{M}$ enzyme, 10 mM EDTA, and $1\ \mu\text{M}$ 5-deazaflavin in 50 mM potassium phosphate, pH 7.0, at 25 °C.

more than a day (Figure 3). Afterward the enzyme was treated with 3% trichloroacetic acid, and the absorbance spectrum of the FAD in the supernatant was identical to that obtained from the native enzyme (Figure 2).

Reduction of the Flavin. Upon initial photoreduction there was an absorbance decrease of the peak at 438 nm, concurrent with an increase around 550 nm due to formation of a flavin semiquinone (Figure 4). Further exposure to light led to a continued decrease around A_{438} and disappearance of the longer-wavelength band, indicating the generation of the two-electron reduced FAD hydroquinone. Subsequent

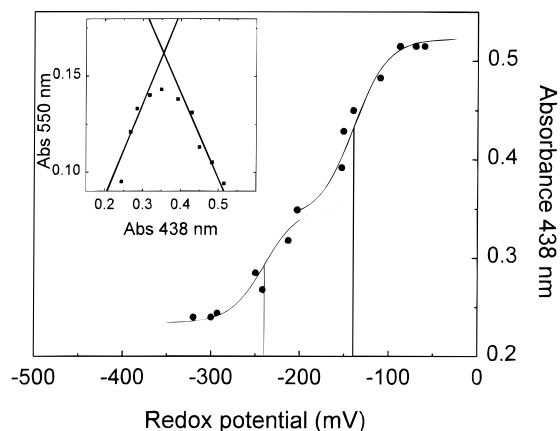


FIGURE 5: Redox titration of 4-hydroxybutyryl-CoA dehydratase with dithionite. Enzyme (11 μ M in 50 mM potassium phosphate, pH 7.0) was reduced with increasing concentrations of dithionite, and the redox potential was measured with an electrode. The two sigmoidal fits are drawn based on the observation that maximal semiquinone is measured at $A_{438} \sim 0.35$ (inset). The redox potentials for $E_{\text{ox/sq}}$ and $E_{\text{sq/red}}$ were estimated to be at -140 and -240 mV (± 15 mV), respectively (both at pH 7.0, 25 $^{\circ}$ C). The inset shows appearance and decrease of semiquinone (A_{550}) plotted against the overall reduction (A_{438}).

irradiation did not yield any additional spectral changes — the broad peak around 420 nm persisted — implying that the Fe-S cluster was not affected under these conditions. Identical results were obtained with either EDTA, glycine, or oxalate as the source of electrons during photoreduction. In general, however, 0.1 M glycine was employed to avoid the possibility of unwanted chelating effects.

The spectral features of the semiquinone are characteristic of a neutral flavin semiquinone (Massey & Palmer, 1966). Typically, those flavoproteins that stabilize the neutral semiquinone do not bind sulfite tightly (Massey et al., 1969). Sulfite-binding can be assayed spectroscopically since it causes a decrease in absorbance similar to that observed upon reduction of the flavin. With dehydratase no spectral changes were observed when the enzyme was reacted with up to 10 mM sulfite. This is consistent with the enzyme stabilizing a neutral rather than anionic semiquinone.

In addition to the method of photoreduction, the flavin could also be reduced with dithionite or with the xanthine/xanthine oxidase system. With all three methods, equilibration occurred within seconds during reduction from oxidized flavin to flavin semiquinone. Further reduction to the flavin hydroquinone required equilibration times of minutes to hours. Besides the routine presence of 5 μ M benzyl viologen ($E_{\text{m7}} = -359$ mV), addition of 2-anthraquinone sulfonate ($E_{\text{m7}} = -225$ mV) or anthraquinone 2,6-disulfonate ($E_{\text{m7}} = -184$ mV) at approximately 1 μ M did not significantly affect these rates.

Determination of Redox Potentials. To determine the midpoint potentials of $E_{\text{ox/sq}}$ and $E_{\text{sq/red}}$, purified dehydratase was titrated with dithionite. The redox potential after equilibration was measured with an electrode, whereas the state of the flavin was observed spectroscopically. The spectral changes of the dehydratase during this titration were essentially analogous to the spectral changes observed during photoreduction (Figure 5). That is, the enzyme was first reduced to the flavin semiquinone followed by reduction to the flavin hydroquinone. The appearance and disappearance of flavin semiquinone are particularly apparent around 550

nm, since there is no absorbance due to oxidized or reduced flavin. However, the absorbance at 550 nm does not lend itself well to determine the redox potential due to the small absorbance changes involved. The largest changes were observed at 438 nm; here the absorbance decreases both for the transition from oxidized flavin to semiquinone and for the transition from semiquinone to fully reduced FAD. In order to evaluate these changes, it was necessary to determine the value of A_{438} at the maximum semiquinone concentration as follows.

The inset of Figure 5 compares the formation and disappearance of semiquinone against overall reduction (A_{550} against A_{438}). Extrapolation of the linear part of the changes gives two lines that intercept at the maximally achievable semiquinone concentration. This is reached when A_{438} is equal to 0.35. Consequently, in the graph of A_{438} against the measured redox potential (Figure 5) two sigmoidal functions are fitted to the data, with a plateau imposed around the determined value for maximal semiquinone ($A_{438} = 0.35$). From this, the redox potentials for $E_{\text{ox/sq}}$ and $E_{\text{sq/red}}$ were estimated to be about -140 mV (± 15 mV) and -240 mV (± 15 mV) respectively. Thus, $E_{\text{ox/red}}$ was calculated to be around -180 mV (all values at 25 $^{\circ}$ C, pH 7.0), which places it in the normal range of redox potentials exhibited by protein bound flavins.

The inset of Figure 5 shows that A_{550} approached about 80% of the theoretical maximum derived from the intercept of the extrapolated lines. This indicates that the enzyme stabilizes the formation of semiquinone to about 80% (the relative stabilization is defined as the actually observed concentration of semiquinone divided by the maximally possible concentration).

For the reaction



there is a correlation between the equilibrium constant, K_{eq} , and the maximally stabilized semiquinone (Clark, 1960):

$$\text{relative stabilization (\%)} = \frac{\sqrt{K_{\text{eq}}}}{2 + \sqrt{K_{\text{eq}}}} \times 100$$

$$E_{\text{ox/sq}} - E_{\text{sq/red}} = 60 \ln K_{\text{eq}} \text{ (mV)}$$

With a stabilization of about 80% the difference between $E_{\text{ox/sq}}$ and $E_{\text{sq/red}}$ was calculated to be around 100 mV, which agrees well with the difference in midpoint potentials determined by curve-fitting A_{438} as a function of redox potential.

EPR Measurements: Flavin Radical. The EPR spectrum of active dehydratase showed essentially no signal at 100 K, as expected for oxidized flavin. When the enzyme was reduced such that the UV/vis absorbance indicated the presence of a flavin semiquinone, a radical signal was measured with $g = 2.0014$ (Figure 6A). The radical signal was readily detectable at 100 K and could be easily saturated at lower temperatures. It showed no hyperfine structure and had a peak-to-peak width of 2 mT. These properties are characteristic for a neutral flavin semiquinone (Palmer et al., 1971). When the FAD was photoreduced further to the hydroquinone, the radical signal disappeared.

Since appearance and decrease of the radical signal correlated completely with the presence of a flavin semi-

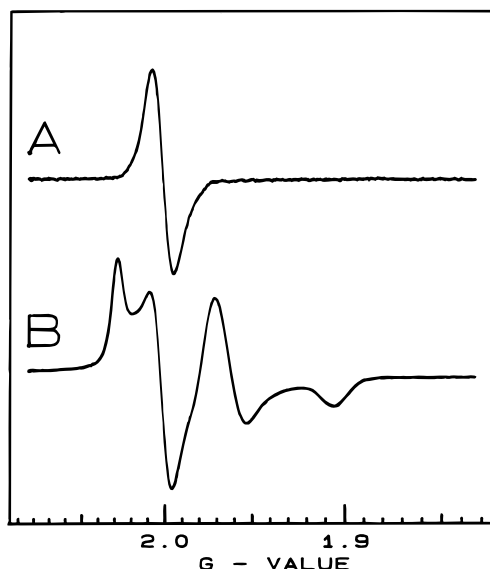


FIGURE 6: EPR spectra of 4-hydroxybutyryl-CoA dehydratase. (A) Enzyme (160 μ M) reduced to the semiquinone: an isotropic signal ($g = 2.0014$) indicative of a radical species appeared. (B) Air-inactivated enzyme (160 μ M): two additional signals appear (total spin concentration is less than 1% of the enzyme concentration) with $g_{xy} = 2.001$, $g_z \approx 2.028$ and $g_{xy} = 1.96$, $g_z = 1.91$. Spectra were measured at a microwave frequency of 9.418 GHz, with a modulation amplitude of 1.27 mT and a modulation frequency of 100 Hz, and at a temperature of 16 K. The power was 2×10^{-4} mW in A and 2 mW in B.

quinone as determined by UV/vis absorbance, it is clear that the signal must be due to the flavin radical. In order to further rule out the possibility that the radical signal was due to an impurity, the presence of the radical signal and the dehydratase activity were monitored during purification of the enzyme. The radical signal could be observed because the enzyme was partially reduced (as confirmed by UV/vis spectroscopy) due to the presence of dithiothreitol and dithionite in the chromatography buffers. At the same time, reductants did not interfere with activity measurements since the assay was performed aerobically. Thus, the enzyme was immediately oxidized in the assay and could initiate catalysis. Throughout purification the specific activity of the enzyme correlated with the relative intensity of the radical signal (not shown).

EPR Measurements: Fe-S Cluster. No EPR signal indicative of an Fe-S cluster was observed in the active enzyme at temperatures down to 4.2 K. This observation, however, does not rule out the presence of an Fe-S cluster, since both $[2\text{Fe-2S}]^{2+}$ and $[4\text{Fe-4S}]^{2+}$ are EPR silent. In order to detect their presence and to distinguish between them, it is necessary to change the redox state of the cluster to a paramagnetic species.

First, it was attempted to oxidize the cluster. Oxidation to a possible $[4\text{Fe-4S}]^{3+}$ cluster, however, was unsuccessful. In the presence of about 10 mM ferricyanide no signal appeared that would indicate a high-potential iron protein (HiPIP)-like Fe-S species (Johnson, 1994, and references therein).

A second approach aimed at generating a $[3\text{Fe-4S}]^+$ cluster. In analogy with the results for aconitase (Emptage et al., 1983a; Kennedy et al., 1983), it was argued that air-inactivation of the enzyme might lead to loss of a "labile" iron atom, resulting in conversion to $[3\text{Fe-4S}]^+$ possibly via a $[4\text{Fe-4S}]^{3+}$ cluster. The dehydratase was exposed to air

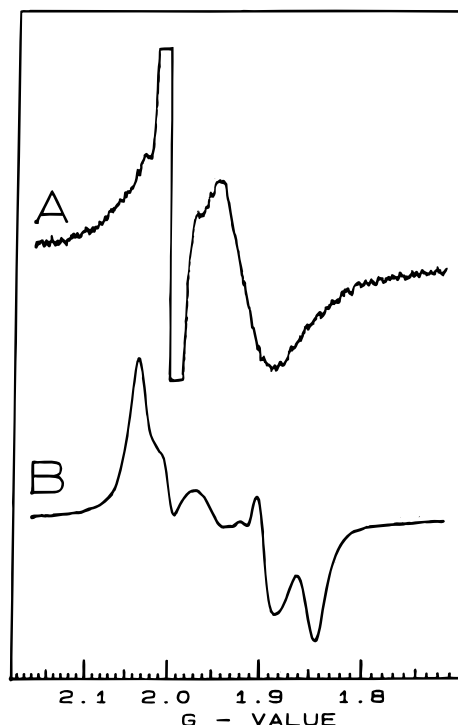


FIGURE 7: Evidence for a reduced $[4\text{Fe-4S}]^+$ cluster. (A) Reduced dehydratase (32 μ M) in 80% DMSO; $g_x = 1.88$, $g_y = 1.93$, and $g_z = 2.032$. The radical signal was omitted for simplification. (B) Reduced dehydratase (160 μ M) in the absence of DMSO; g -values of 2.037, 1.895, and 1.844. The reactions contained 50 μ M 5-deazaflavin in 50 mM potassium phosphate, pH 7.0, and were irradiated for 45 min. Spectra were measured at 2 mW, with a modulation amplitude of 1.27 mT and a modulation frequency of 100 Hz. Microwave frequency was 9.424 GHz (A) or 9.421 GHz (B), and the temperature was 10 K (A) or 12 K (B).

at 0 °C until the activity had dropped to less than 10% of the starting value (Figure 3). New signals appeared (Figure 6B): an axial signal with all g -values greater than 2 ($g_{xy} = 2.001$, $g_z = 2.028$) and an axial signal with all g -values smaller than 2 ($g_{xy} = 1.96$, $g_z = 1.91$). However, these signals amounted to less than 0.02 spin/mol and were not typical of a $[3\text{Fe-4S}]^+$ cluster. The same signals were observed when the enzyme was purified in the absence of reducing agents, resulting in a smaller specific activity. The signals were therefore not considered to belong to the active enzyme. Possibly they are a consequence of protein denaturation.

Thirdly, it was attempted to reduce the Fe-S cluster. On the basis of the observations with UV/vis spectroscopy, it was already clear that this would be difficult. A rather forceful approach involved the treatment with dimethyl sulfoxide (DMSO). It has been reported that DMSO can loosen protein folding to the extent that previously buried Fe-S clusters may become accessible to reducing agents (Cammack, 1973). Addition of 80% DMSO, followed by photoreduction in the presence of 50 μ M 5-deazariboflavin indeed led to the appearance of a broad, slightly rhombic signal (Figure 7A), indicative of an unstrained cubane Fe-S cluster with $g_x = 1.88$, $g_y = 1.93$, and $g_z = 2.032$. It can be argued, however, that this observation is not relevant for the native state of the enzyme. The cluster may have formed spontaneously upon partial unfolding of the peptide chain.

Extensive photoreduction in the absence of DMSO but again with high concentrations of catalyst (50 μ M 5-deazariboflavin) finally yielded a signal typical for $[4\text{Fe-4S}]^+$ with

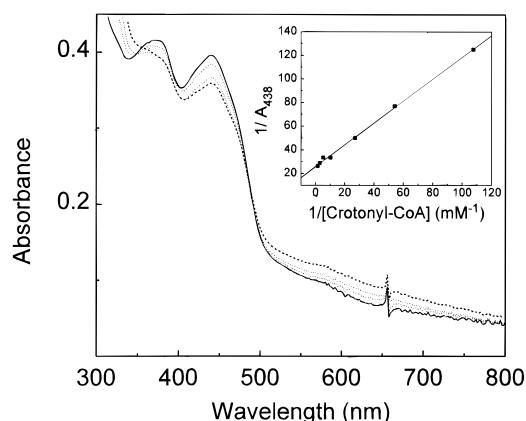


FIGURE 8: Spectrum of the dehydratase in the presence of crotonyl-CoA. Oxidized enzyme (solid line; 8 μ M in 50 mM potassium phosphate, pH 7.0, 25 $^{\circ}$ C) was incubated with 1.9, 98, and 640 μ M crotonyl-CoA (dotted lines). The inset shows the double-reciprocal plot of the absorbance changes at 438 nm against crotonyl-CoA concentration. From this the K_d is calculated to be 40 μ M.

g-values equal to 2.037, 1.895, and 1.884 (Figure 7B). At higher temperatures the signal started to broaden, which is again characteristic of a $[4\text{Fe-4S}]^+$ cluster. The spin concentration was determined to be around 0.2 spin/mol of enzyme. However, during irradiation of the sample, it was visually apparent that only a fraction of the enzyme in the EPR tube was bleached (reduced) at any given time. If the sample was removed from the light source, the brown color immediately reappeared. The gas phase over the sample had been exchanged by repeated evacuation and flushing with argon. Thus, inadequate anaerobiosis should not have been the case, but cannot be ruled out. Nevertheless, the observation of only partial reduction presumably accounts for the relatively low yield in spin concentration.

Reaction of the Reduced Enzyme with Oxygen. It was initially observed that enzyme reduced to the flavin hydroquinone reoxidizes in the presence of air within mixing time. To quantitate the kinetics of reoxidation, dehydratase was reduced by photoreduction or with the xanthine oxidase system and then reacted with varied concentrations of oxygen. The rate of reoxidation was recorded with the stopped-flow apparatus and showed multiphase kinetics. The reason for this was not further pursued. It can be stated, however, that in the presence of 0.5 mM crotonyl-CoA none of the limiting rates was higher than 0.1 s^{-1} at 25 $^{\circ}$ C. This is consistent with the observation that the enzyme assay can be performed under aerobic conditions. With a V_{max} of 24 s^{-1} , oxygen does not interfere significantly with the reaction in the presence of high substrate concentrations.

Spectral Properties of the Dehydratase in Equilibrium with Substrate. Substrate-dependent changes were measured in all cases by reaction with crotonyl-CoA since it is much more stable than 4-hydroxybutyryl-CoA and the equilibrium constant is around 4 on the side of crotonyl-CoA (Scherf & Buckel, 1993). Upon incubation of dehydratase with crotonyl-CoA there were distinct spectral changes. The peak at 438 nm decreased, and a long-wavelength band appeared in the region from 500 to 800 nm (Figure 8). While the change at 438 nm was reproducible with all enzyme preparations, the intensity of the longer-wavelength band varied with enzyme preparation. For dehydratase with high specific activity (>5 units/mg) the absorbance changes were large

enough to allow titration with the product (inset of Figure 8). A dissociation constant of around 40 μ M was derived from the plot. Interestingly, the spectral changes were relatively stable to oxygen. Upon exposure to air, the long-wavelength band decayed with a k_{obs} of $3 \times 10^{-4} \text{ s}^{-1}$ (not shown).

To test whether the same spectral changes could be achieved with 4-hydroxybutyryl-CoA, 30 μ M dehydratase was incubated with 4 mM acetyl-CoA and 40 mM 4-hydroxybutyrate. Neither reactant affected the spectrum. After addition of 3 units of 4-hydroxybutyrate CoA transferase, however, the absorbance changes reflected those due to the presence of crotonyl-CoA (not shown).

DISCUSSION

UV/Vis Absorbance. The spectral features of 4-hydroxybutyryl-CoA dehydratase are typical of an iron-sulfur flavoprotein. The UV/vis absorbance spectrum clearly shows the contribution of the FAD with well-defined peaks at 438 nm and at 380 nm, as well as the absorbance by the Fe-S clusters. The latter has a broad maximum around 420 nm. It should be noted that this is only a first approximation of the Fe-S spectrum, since it was derived by subtracting the spectrum of the released flavin from that of the holoenzyme. This method does not take into account any changes of the flavin absorbance due to binding to the protein. Such changes are generally small. The difference spectrum is in fact quite similar to the spectrum published for glutamine phosphoribosyl amidotransferase (Wong et al., 1977; Oñate et al., 1989), and for active aconitase (Emptage et al., 1983a). Both enzymes contain an Fe-S cluster without a function in electron transfer. The origin of the broad and sloping background absorption notably increasing in the near-UV is not understood.

Stabilization of the Flavin Semiquinone. The active enzyme contains oxidized flavin that can be reduced sequentially to the semiquinone and to the hydroquinone ($E_{\text{ox/sq}} = -140 \text{ mV}$; $E_{\text{sq/red}} = -240 \text{ mV}$, at 25 $^{\circ}$ C, pH 7.0, respectively). The protein stabilizes the neutral form of the semiquinone, which is characterized by an absorbance band that stretches beyond 550 nm (Massey & Palmer, 1966). The protonated character of the flavin semiquinone is confirmed by the shape and linewidth of the EPR signal. Moreover, the enzyme does not bind sulfite. Tight binding of sulfite has been described as a diagnostic quality only for those flavoproteins that stabilize the anionic flavin radical (Massey et al., 1969). Finally, the amino acid sequence of the dehydratase indicates significant similarities to two flavoprotein hydroxylases (Gerhardt, 1995), a class of enzymes that typically stabilize the neutral semiquinone (Massey et al., 1969).

Structural Properties of the Fe-S Cluster. The active dehydratase was EPR silent under conditions appropriate for observing most Fe-S clusters. After extensive photoreduction, an EPR signal indicative of a $[4\text{Fe-4S}]^+$ cluster was observed, implying that the dehydratase contained a $[4\text{Fe-4S}]^{2+}$ cluster prior to reduction. This inference is consistent with our initial failure to observe an EPR signal, since $[4\text{Fe-4S}]^{2+}$ clusters are EPR silent.

It was very difficult to reduce the Fe-S cluster. This observation is reminiscent of other Fe-S proteins with a non-redox role. In aconitase the only successful reduction was

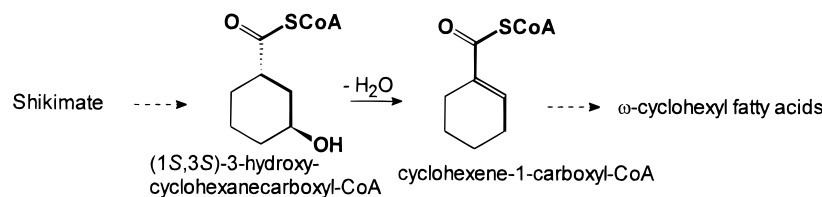


FIGURE 9: Dehydration of (1S,3S)-3-hydroxycyclohexanecarboxyl-CoA, a step analogous to the dehydration of 4-hydroxybutyryl-CoA in the biosynthesis of ω -cyclohexyl fatty acids in *A. acidocaldarius*.

achieved by photoreduction in the presence of 5-deazaflavin (Emptage et al., 1983b). The same was found for enzymes that are not hydrolyases and may contain [4Fe-4S] clusters for structural stabilization, such as glutamine phosphoribosylpyrophosphate-amidotransferase (Vollmer et al., 1983; Oñate et al., 1989) and endonuclease III (Cunningham et al., 1989).

Involvement of Flavin and Fe-S in Catalysis. It is presumed that the Fe-S cluster in 4-hydroxybutyryl-CoA dehydratase has a structural function and/or serves as a Lewis acid during catalysis. Evidence for the latter would have been the conversion of [4Fe-4S]²⁺ to [3Fe-4S]⁺ upon inactivation with air as has been reported for aconitase (Kent et al., 1982; Kennedy et al., 1983). This would have indicated the presence of an iron atom in the cluster that is not liganded by a protein residue; such an iron atom would be available as a ligand to the substrate during catalysis and would presumably be relatively labile to air. However, no EPR signal attributable to a [3Fe-4S]⁺ cluster was observed. Apart from the appearance of two minor signals, the EPR spectrum of air-inactivated enzyme is essentially the same as that of active enzyme. It is not clear, therefore, how air inactivates the enzyme or whether the [4Fe-4S]²⁺ cluster could function as a Lewis acid.

It seems very likely that the Fe-S cluster does not play a redox role in catalysis, given that it is difficult to reduce and that there is no equilibration of electrons between the FAD and the Fe-S cluster. This makes 4-hydroxybutyryl-CoA dehydratase different from other proteins that contain both a flavin and an Fe-S cluster [for a recent review see Johnson (1994)]. The redox behavior is, however, consistent with the proposed role for the FAD which probably undergoes transient reduction and oxidation during turnover to enable dehydration of the substrate. Putative cycling of the FAD between the oxidized and the one-electron reduced state is supported by the observation that transition between these two oxidation states is fast, whereas reduction to the hydroquinone appears to be kinetically hindered. A one-electron cycle would be consistent with the mechanism proposed in Figure 1A.

Involvement of the flavin in turnover is further supported by the spectral changes of the enzyme upon incubation with crotonyl-CoA, namely, a decrease in the peak at 438 nm and the appearance of a broad absorption band from 500 to 800 nm. Enzyme preparations with higher specific activities showed a more pronounced appearance of this long-wavelength absorbance. Less active enzyme showed merely the changes at 438 nm. This points toward the absorbance at 500–800 nm representing a catalytically significant species.

There are at least two plausible explanations for the long-wavelength absorption. It could indicate a charge transfer interaction between substrate (or intermediate) and flavin. Alternatively, it could be due to a flavin semiquinone species

being present in the enzyme–substrate equilibrium. In principle, EPR measurements should help distinguish between flavin semiquinone and a charge transfer interaction, since the former will give a radical signal whereas the latter will not. Upon incubation with crotonyl-CoA, the enzyme did not show a significant increase in the radical signal. This may indicate that indeed the band is due to a charge transfer interaction, which would also agree with the observation that the absorbance changes at 438 and 550 nm do not correlate. A possible interpretation would be that in less active enzyme preparations, the substrate is still bound (i.e., A_{438} decreases) but does not interact optimally with the flavin. Hence, the charge transfer complex is no longer formed and the rate of turnover is reduced. Alternately, it cannot be ruled out that the long-wavelength absorbance is due to a flavin semiquinone which is not detectable by EPR. According to the mechanism postulated in Figure 1A, the flavin radical is generated concurrently with a substrate radical. If the two species are in close proximity, the two single electrons may couple and appear EPR silent. This interpretation is supported by the observation that upon photoreduction of the enzyme to semiquinone, the intensity of the long-wavelength band also correlates with the specific activity. In less active dehydratase, the band around 550 nm is less pronounced.

Vinylacetyl-CoA Isomerization. Interestingly, inactivated enzyme is still able to catalyze the isomerization of vinylacetyl-CoA to crotonyl-CoA, albeit at a slower rate than active enzyme. Isomerization can be simply drawn as deprotonation and stabilization of the carbanion at C-2 and reprotonation at C-4 (Figure 1B). It seems that the air-exposed enzyme has retained the ability of α -proton abstraction, while it is no longer able to undergo the more complicated chemistry of 4-hydroxybutyryl-CoA turnover. These results imply, however, that the Fe-S cluster, which one would expect to be destroyed by oxygen, is somehow involved in the catalysis of dehydration, although the EPR data do not support this view. Therefore other methods, e.g., Mössbauer spectroscopy, should be applied to resolve this contradiction. The stability of the isomerase activity under air is consistent with the original observation by Stadtman and co-workers who investigated fermentation by *C. aminobutyricum* (Hardman & Stadtman, 1963). They were able to measure isomerase activity in the cell extract but never any dehydration activity. Most likely this was due to the fact that the cells had been broken aerobically, and consequently the 4-hydroxybutyryl-CoA dehydratase was inactivated yet had retained the isomerization ability.

Biosynthesis of Cyclohexanecarboxylate. H. G. Floss informed the authors that a reaction closely related to the dehydration of 4-hydroxybutyryl-CoA is most probably involved in the biosynthesis of the cyclohexanecarboxylate moiety of the antibiotic ansatrienine from shikimate in *Streptomyces collinis* (Moore et al., 1993a). Furthermore, *Alcyclobacillus acidocaldarius* contains ω -cyclohexyl fatty

acids in the cytoplasmic membrane. A mutant strain, auxotrophic for cyclohexanecarboxylate, accumulated (1S,3S)-3-hydroxycyclohexanecarboxylate, which is thought to be dehydrated to cyclohexene-1-carboxylate, most likely on the CoA-ester level (Moore et al., 1993b) (Figure 9). This reaction, in which several restrictions on the stereochemistry are involved, is completely consistent with the proposed mechanism (Figure 1A). In the substrate both the α -hydrogen and the γ -hydroxyl group can occupy the more favorable equatorial positions, and the α,β -double bond in the product has (*E*)-conformation as in crotonyl-CoA.

Conclusion. It was shown that Fe and S in 4-hydroxybutyryl-CoA dehydratase form a $[4\text{Fe-4S}]^{2+}$ cluster which is difficult to reduce and not converted to an EPR active $[3\text{Fe-4S}]^+$ cluster during inactivation by oxygen. It is therefore likely to have a structural function. In addition it could be involved in substrate binding but not in electron transfer. The properties of the flavin, however, make it ideally suited as a transient sink for one-electron reduction. An FAD_{ox} to FAD_{sq} transition is quite possible and would be consistent with the ketyl-radical mechanism postulated in Figure 1A. Although direct evidence for a ketyl-radical was not obtained, such an intermediate serves as an intriguing working hypothesis and may well be relevant for other dehydrations (Buckel & Keese, 1995).

ACKNOWLEDGMENT

We thank Iris Schall, Philipps-Universität Marburg, for her excellent technical assistance in cultivation of the bacteria and in protein purification.

REFERENCES

- Albracht, S. P. J. (1980) *Biochim. Biophys. Acta* 612, 11–28.
- Beinert, H., & Kennedy, M. C. (1989) *Eur. J. Biochem.* 186, 5–15.
- Buckel, W. (1992) *FEMS Microbiol. Rev.* 88, 211–232.
- Buckel, W., & Keese, R. (1995) *Angew. Chem., Int. Ed. Engl.* 34, 1502–1506.
- Cammack, R. (1973) *Biochem. Biophys. Res. Commun.* 54, 548–554.
- Clark, W. M. (1960) *Oxidation–Reduction Potentials in Organic Systems*, Robert E. Krieger Publishing Company, Huntington, NY.
- Cunningham, R. P., Asahara, H., Bank, J. F., Scholes, C. P., Salerno, J. C., Surerus, K., Münck, E., McCracken, J., Peisach, J., & Emptage, M. H. (1989) *Biochemistry* 28, 4450–4455.
- Emptage, M. H. (1988) in *Metal Clusters in Proteins* (Que, L., Ed.) ACS Symposium Series 372, pp 343–371, American Chemical Society, Washington, DC.
- Emptage, M. H., Dreyer, J. L., Kennedy, M. C., & Beinert, H. (1983a) *J. Biol. Chem.* 258 (18), 11106–11111.
- Emptage, M. H., Kent, T. A., Kennedy, M. C., Beinert, H., & Münck, E. (1983b) *Proc. Natl. Acad. Sci. U.S.A.* 80, 4674–4678.
- Flint, D. H., & Emptage, M. H. (1988) *J. Biol. Chem.* 263, 3558–3564.
- Flint, D. H., Emptage, M. H., Finnegan, M. G., Fu, W., & Johnson, M. (1993a) *J. Biol. Chem.* 268, 14732–14742.
- Flint, D. H., Smyk-Randall, E., Tuminello, J. F., Draczynska-Lusiak, B., & Brown, O. R. (1993b) *J. Biol. Chem.* 268, 25547–25552.
- Gerhardt, A. (1995) Masters Thesis, Philipps Universität Marburg, Germany.
- Grabowski, R., Hofmeister, A. E. M., & Buckel, W. (1993) *Trends Biochem. Sci.* 18, 297–300.
- Grandoni, J. A., Switzer, R. L., Makaroff, C. A., & Zalkin, H. (1989) *J. Biol. Chem.* 264, 6058–6064.
- Hardman, J. K., & Stadtman, T. C. (1963) *J. Biol. Chem.* 238, 2088–2093.
- Hofmeister, A. E. M., Albracht, S. P. J., & Buckel, W. (1994) *FEBS Lett.* 351, 416–418.
- Johnson, M. K. (1994) in *Encyclopedia of Inorganic Chemistry* (King, R. B., Ed.) Vol. 4, pp 1896–1915, Wiley, U.K.
- Kennedy, M. C., Emptage, M. H., Dreyer, J. L., & Beinert, H. (1983) *J. Biol. Chem.* 258, 11098–11105.
- Kent, T. A., Dreyer, J. L., Kennedy, M. C., Huynh, B. H., Emptage, M. H., Beinert, H., & Münck, E. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 1096–1100.
- Massey, V. (1991) in *Flavins and Flavoproteins* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 59–66, Walter de Gruyter, Berlin, New York.
- Massey, V., & Palmer, G. (1966) *Biochemistry* 5, 3181–3189.
- Massey, V., & Hemmerich, P. (1977) *J. Biol. Chem.* 252, 5612–5614.
- Massey, V., & Hemmerich, P. (1978) *Biochemistry* 17, 9–17.
- Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., & Foust, G. P. (1969) *J. Biol. Chem.* 244, 3999–4006.
- Molander, G. A., & Hahn, G. (1986) *J. Org. Chem.* 51, 1135–1138.
- Moore, B. S., Cho, H., Casati, R., Kennedy, E., Reynolds, K. A., Mocek, U., Beale, J. M., & Floss, H. G. (1993a) *J. Am. Chem. Soc.* 115, 5254–5266.
- Moore, B. S., Poralla, K., & Floss, H. G. (1993b) *J. Am. Chem. Soc.* 115, 5267–5274.
- Oñate, Y. A., Vollmer, S. J., Switzer, R. L., & Johnson, M. K. (1989) *J. Biol. Chem.* 264, 18386–18391.
- Palmer, G., Müller, F., & Massey, V. (1971) in *Flavins and Flavoproteins* (Kamin, H., Ed.) pp 123–140, University Park Press, Baltimore, MD.
- Robbins, A. H., & Stout, C. D. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 3639–3643.
- Scherf, U., & Buckel, W. (1991) *Appl. Env. Microbiol.* 57, 2699–2702.
- Scherf, U., & Buckel, W. (1993) *Eur. J. Biochem.* 215, 421–429.
- Scherf, U., Söhling, B., Gottschalk, G., Linder, D., & Buckel, W. (1994) *Arch. Microbiol.* 161, 239–245.
- Simon, E. J., & Shemin, D. (1953) *J. Am. Chem. Soc.* 75, 2520.
- Switzer, R. L. (1989) *BioFactors* 2, 77–86.
- Vollmer, S. J., Switzer, R. L., & Debrunner, P. G. (1983) *J. Biol. Chem.* 258, 14284–14293.
- Whitby, L. G. (1953) *Biochem. J.* 54, 437–445.
- Willadsen, P., & Buckel, W. (1990) *FEMS Microbiol. Lett.* 70, 187–192.
- Wong, J. Y., Meyer, E., & Switzer, R. L. (1977) *J. Biol. Chem.* 252, 7424–7426.

BI9601363