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Studies on the reaction mechanism of general acyl-CoA dehydrogenase

Determination of selective isotope effects in the dehydrogenation of butyryl-CoA

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The kinetic properties of general acyl-CoA dehydrogenase from pig kidney have been investigated using normal butyryl-CoA as well as a α-deutero, β-deutero- and perdeutero-butyryl-CoA. In turnover catalysis, isotope effects of 2, 3.6, and 9 were found respectively. In the reductive half reaction the isotope effects were 2.5, 14, and 28 for the same substrates, and 21 for (2*R*,3*R*)-(2,3-D_2)butyryl-CoA. No intermediates are apparent during the reduction of oxidized enzyme to the presumed complex of reduced enzyme and crotonyl-CoA. The results are interpreted as indicating a high degree of concertedness during the rupture of the α and β C-H bonds. They are compatible with a mechanism in which simultaneously the α-hydrogen is abstracted as a proton, while the β-hydrogen is transferred to the oxidized flavin as a hydride.

The mechanism of acyl-CoA dehydrogenases is of particular chemical interest, since it involves the rupture of two kinetically stable C-H bonds [1–3]. The overall reaction might be envisaged as occurring by a variety of mechanisms such as via transient carbanions, by radical processes, via hydride transfer, via covalent intermediates or by a combination of these routes. These aspects have been reviewed recently elsewhere [4]. Of particular interest is also the question whether the rupture of the α and β C-H bonds occurs in a synchronous/concerted way or via defined intermediates, i.e. by sequential, kinetically differentiable steps. Several attempts to provide answers to these questions have involved the use of spectroscopic reaction kinetic techniques often combined with the use of deuterated substrates. Murfin [5] and, later, Reinsch et al. [6] described unusually large isotope effects for the oxidation of protio/perdeutero butyryl-CoA by pig liver 'general' acyl-CoA dehydrogenase; the latter authors suggested that dehydrogenation might involve hydrogen tunneling and also reported the reaction as being essentially irreversible. In contrast to this, results from kinetic studies by Raichle [7] using the bacterial enzyme butyryl-CoA dehydrogenase were consistent with a system in which reduction is freely reversible. With respect to the chemical mechanism of fatty-acyl-CoA dehydrogenase, McFarland and collaborators [6, 8] have proposed that (charge-transfer) complexes between the carbanion derived from substrate deprotonation at position 2 and the oxidized flavin are obligatory intermediates in catalysis. More recently Ikeda et al. [9] have put forward a rather peculiar mechanism, involving a 'donor-acceptor resonance hybrid' in which a hydrogen is proposed to be shared between the substrate and the flavin N(5) position. These authors [9] also propose, that 'hydride ion transfer is not yet complete in the charge transfer complex', which is formed upon reaction of the oxidized enzyme with substrate, essentially implying that this hydride-sharing species is a stable entity. Clearly these proposals are in contrast with the findings of Murfin [5], the results from our groups [7, 10] and by Gomes et al. [11]. In view of these inconsistencies we have set out to investigate some mechanistic details of the flavin-catalyzed α,β-dehydrogenation using the general acyl-CoA dehydrogenase (GAD) from pig kidney. This protein has the advantage that it is readily available in considerable quantities, has a broad specificity [12], is stable and is essentially free of ligands which have been found to bind tightly and to induce long-wavelength (charge-transfer) absorptions in the enzymes from other sources [13, 14].

MATERIALS AND METHODS

Materials

General acyl-CoA dehydrogenase was isolated from pig kidney as described by Thorpe et al. [12, 15]. *n*-Butyryl-CoA, lithium salt, was from P-L Biochemicals (Milwaukee, WI). Deuterated butyric acids were from Fa. Roth Feinchemikalien (Karlsruhe, FRG). Deuterated butyryl-CoA derivatives were prepared from the free acids and CoA-SH by the method of Goldmann and Vagelos [16]. The products were purified by HPLC (RP-18, Merck) using a 0.02 M sodium phosphate buffer, pH 6.0/methanol gradient (15–30% methanol); butyryl-CoA elutes at 23–25% methanol. (2*R*,3*R*)-(2,3-D_2)Butyryl-CoA was prepared as described earlier [10]. The purified CoA derivatives were separated from inorganic salt by chromatography over a P2 (BioRad) column in distilled water. The purity of the deuterated butyryl-CoA derivatives prepared by this method was determined by NMR spectroscopy and analytical HPLC in the system described above. In no case was evidence found for exchange of

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Abbreviations. GAD, general acyl-CoA dehydrogenase; HPLC, high-pressure liquid chromatography; D, deuterium (^{2}H).

Enzyme. General acyl-CoA dehydrogenase (EC 1.3.99.3).

deuterium, the isotopic purity being >97% and the butyryl-CoA content being >95%.

Instrumentation

Absorption spectra were measured with a Kontron Uvikon 820 spectrophotometer specially equipped for measurements at $t < 0\,°C$ (N_2-flushing of quartz cell). The stopped-flow instrument used was constructed as described elsewhere [7]; it was equipped with a scan recorder (Princeton Applied Research, model 4101 A). Alternatively the data acquisition was done with an Apple II microcomputer equipped with a fast A/D converter (Interactive Structures AI13), using software which will be described elsewhere (Boschert, W., Ph.D. thesis, University of Konstanz, unpublished). NMR-spectra were recorded with a Bruker FT 250 instrument. HPLC was done using Altex or Kontron pumps and a Kontron Anacomp controller/integrator system. Computer simulations were done with either a Hewlett Packard desk computer model 9820 [7] or with an Apple II microcomputer (Boschert W. and Ghisla, S., unpublished).

Methods

Concentration of GAD was determined using an absorption coefficient of 15400 M^{-1} cm^{-1} [12], which refers to enzyme-bound FAD. The absorption of reduced enzyme at 446 nm used for the calculation of the data of Fig. 7 was taken as about 10% that of oxidized GAD and corresponds to that observed upon reduction of the enzyme with dithionite as described by Thorpe et al. [12]. Assays of enzyme activity were carried out essentially as described by Thorpe et al. [12] using phenazine methosulfate (or phenazine ethosulphate) as mediator, and dichloroindophenol as electron acceptor in 0.02 M phosphate buffer, pH 7.6, and by monitoring the decrease of absorbance at 600 nm. The enzyme used for kinetic measurements has typically a turnover number of 40 min^{-1} and 130 min^{-1} using butyryl-CoA and octanoyl-CoA, respectively.

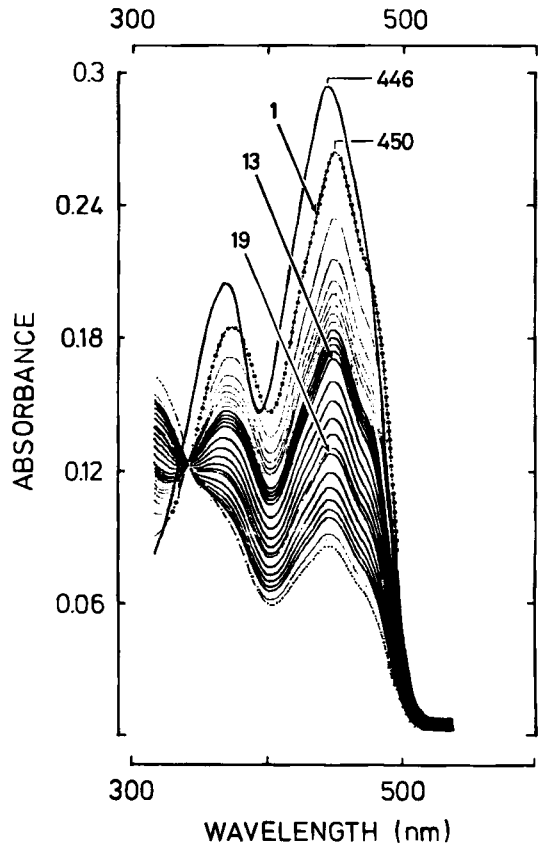


Fig. 1. *Spectral course of the anaerobic reaction of general acyl-CoA dehydrogenase with perdeutero-butyryl-CoA.* A solution of the enzyme, 19.2 μM in 1.0 ml 50 mM phosphate buffer pH 7.2, was adjusted to pH 6.1 with 2 M acetic acid. It was made anaerobic and then reacted at ≈0 °C with 0.2 μmol perdeuterobutyryl-CoA in the same buffer. Recording of the spectra in the range shown was started immediately. The 445-nm point was reached after 11 s in the first scan (curve 1). In curve 2, 445 nm was reached after 47 s. The successive curves, until curve 6, were obtained at 36-s intervals each. Curve 7 was after 3.9 min and curves 8–12 at 0.6-min intervals. Curve 13 was after 8.8 min and curves 14–18 at 3-min intervals. Curve 19 was after 24.5 min and the remaining traces were at 5-min intervals

RESULTS AND DISCUSSION

Spectral course of the reaction of general acyl-CoA dehydrogenase with the substrate butyryl-CoA

Aerobic or anaerobic reaction of the enzyme with various substrates causes a very rapid bleaching of the typical absorption of the enzyme-bound oxidized flavin and, concomitantly, a long-wavelength band extending beyond 700 nm is formed [12]. With the best substrate, octanoyl-CoA, the process is completed in the millisecond time range at 25 °C. We have succeeded in dramatically slowing down this reaction by using fully deuterated butyryl-CoA (D_3C-CD_2-CD_2-COS-CoA) at −3 °C to −5 °C and at pH ≈6. Under these conditions the whole process could be followed by rapid scanning with a conventional spectrophotometer in the range 320–520 nm, as shown in Fig. 1.

The reaction is multiphasic. First a very rapid perturbation of the spectrum of the oxidized flavin occurs, which is similar to that observed upon binding of nonreacting, saturated acyl-CoA derivates [14]. This first-formed species is thus attributed to formation of the Michaelis complex between oxidized GAD and butyryl-CoA (Fig. 1, curve 1). Following this, the reaction proceeds with a clear cut isosbestic point at 342 nm and the changes in the 320–500-nm region occur in three phases. The first two phases are not well separated from each other in this type of experiment. This contrasts with the third, final phase, which is much slower ($t_{1/2}$ ≈11 min at ≈0 °C) and yields a final spectrum which is identical to that obtained in static titrations using normal butyryl-CoA at 25 °C [12]. At $\lambda > 520$ nm the absorbance first increases and then decreases in two phases, the general kinetic behaviour being qualitatively similar to that observed at 450 nm.

These experiments clearly establish two points. Since the kinetic course of the spectral changes follows the same pattern in the wavelength range 320–500 nm and appears to be isosbestic at 342 nm, neither the semiquinone nor other reaction intermediates are spectrally detectable during the reduction of the flavin. Intermediates such as flavin semiquinones (radicals) have been implied by others [17] to occur during the reaction of pig liver acyl-CoA dehydrogenase with octanoyl-CoA and other substrates. Flavin radicals have typical, strong absorbance in the 380–400-nm region as well as at longer wavelengths and are sufficiently different from the spectra of oxidized and fully reduced flavin to be transiently observed at concentrations as low as 5%. Other types of intermediates, such as covalent intermediates, or charge transfer complexes

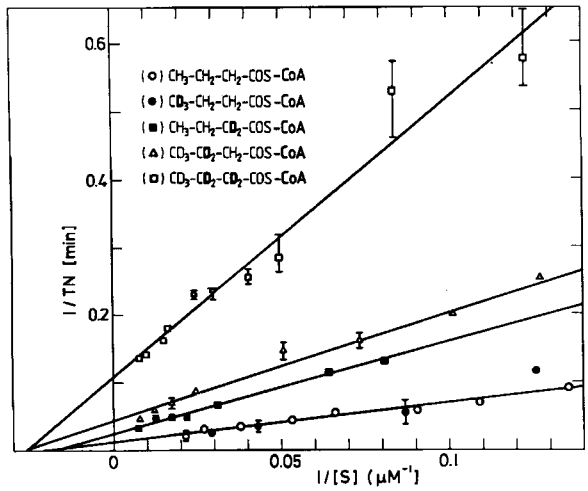


Fig. 2. *Turnover studies of GAD with selectively deuterated butyryl-CoA substrates.* The enzyme activities were measured as detailed in Materials and Methods using the substrates shown in the insert at the concentrations shown. The points are the average of two or three determinations and the bars indicate the experimental scatter for some representative concentrations. The lines shown were determined by least-square-fit analysis of the data points. TN = turnover number

Table 1. *Kinetic constants for turnover of GAD with selectively deuterated butyryl-CoA*

R in substrate R-CO-SCoA	V_{max}	$V_{max}(H)/V_{max}(D)$	K_m
	min^{-1}		μM
CH_3-CH_2-CH_2-	80	–	45
CD_3-CH_2-CH_2-	80	1	45
CH_3-CH_2-CD_2-	40	2	50
CD_3-CD_2-CH_2-	22	3.6	35
CD_3-CD_2-CD_2-	9	9	40

between oxidized flavin and substrate α-carbanions, which have been proposed by others [6, 9], if they occur, must be in very low concentration. On the other hand, the spectral changes are consistent with direct reduction of the oxidized enzyme by the bound butyryl-CoA to form GAD_{red} with crotonyl-CoA still bound. This latter complex is characterized by a long-wavelength absorption [12].

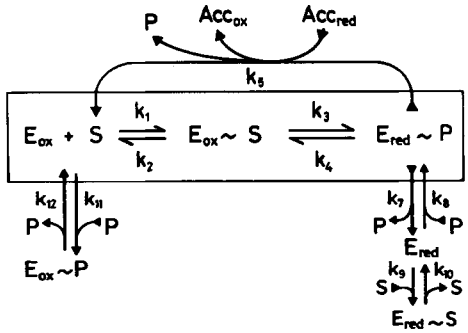
The triphasic nature of this reaction will not be considered further in this paper and will be dealt with in a forthcoming work (Schopfer, L., Ghisla, S., Massey, V. and Thorpe, C., unpublished).

Steady-state kinetics using undeuterated, selectively deuterated and perdeutero-butyryl-CoA

Turnover assays were carried out by the method of Thorpe et al. [12] under the conditions outlined in Fig. 2 and Materials and Methods. The results obtained from the plots of Fig. 2 are listed in Table 1. It should be pointed out that, as reported by Williamson and Engel [18], V_{max} values obtained using dichloroindophenol as electron acceptor and phenazines as mediator can be subjected to considerable experimental variation, in particular when phenazine methosulphate is used. In our case the variation of V_{max} using different enzyme preparations and butyryl-CoA varied maximally between 75 min^{-1} and 85 min^{-1}. The scatter of data observed using phenazine ethosulfate was indeed substantially smaller than with methosulphate, but the V_{max} values were also approximately half those observed using the latter mediator. Furthermore the isotope effects were much smaller, indicating that the rate of electron transfer from reduced GAD to the acceptor (k_5, Scheme 1) becomes limiting with this acceptor. The extrapolated K_m values for the butyryl-CoA derivatives are in the range reported by others and also similar to those obtained with acyl-CoA dehydrogenases from other sources [17–20]. The magnitude of the isotope effects found on V_{max} (Table 1) is smaller than that found for the reduction of the enzyme flavin (k_{obs}) to be detailed below (Table 2). This is in accordance with the finding of smaller isotope effects using phenazine ethosulphate and implies, as already suggested by Beinert [21], that a different step, probably a process involving reoxidation (k_5, Scheme 1) is partially rate-limiting. The same behaviour was found with the dehydrogenase from ox liver studied by Murfin [5] and with bacterial butyryl-CoA dehydrogenase [7]. According to Northrop [22] the ratio of the rates of reduction (k_3, Scheme 1) and of a further (partially) rate-limiting step, in our case the reaction with the electron acceptor and product dissociation (k_5), is reflected by the relation:

$$f_v = \frac{v_H/v_D - 1}{k_H/k_D - 1}$$

where v is the rate of turnover, k is the rate constant of the step in which the C-H/D bond is broken, and f_v is the fractional reduction of the maximal velocity of reaction with normal substrate attributed to breaking of the C-H/D bond. In our case, using the values from Tables 1 and 2, f_v varies from 0.2 to 0.7. This variation is likely to result from an isotope effect on step k_5. This assumption is reasonable since the substrate β-H (or D), which is transferred to the flavin-N(5) position [10] does not exchange with solvent [10]. Rupture of the resulting flavin N(5)-H/D bond occurs during oxidation. A conservative interpretation is that k_3 is of the same order of magnitude as k_5, i.e. that both are rate-limiting on turnover.



Scheme 1. *Minimal scheme proposed to represent the reactions of GAD with butyryl-CoA substrates.* This scheme is an extension of that proposed earlier by Stein-Parvé et al. [1] and later by Thorpe et al. [12]. S = substrate (butyryl-CoA), P = product (crotonyl-CoA) and Acc = acceptor

Pre-steady-state kinetics: stopped-flow study of enzyme reduction half reaction

Fig. 3A illustrates a typical time course of the enzyme reduction using normal butyryl-CoA under anaerobic conditions, at 25 °C and at pH 7.6. Apart from the expected

Table 2. *Rate constants for the reaction of GAD with selectively deuterated butyryl-CoA substrates*
In expts 1–3, k_3 and k_4 were estimated from k_{obs} ($= k_3 + k_4$) and from the endpoint of the reaction, which reflects the ratio k_3/k_4 (cf. text for the details, and Scheme 1 for attribution of k_3 and k_4). In expt 4, k_3 and k_4 were estimated from Fig. 5. In expt 5, the rate was determined at a single concentration of 1 mM (2*R*,3*R*)-(2,3-D_2)butyryl-CoA, and the isotope effect was obtained by comparison with the rate measured using the same concentration of non-deuterated butyryl-CoA

Expt	R for substrate R-COSCoA	k_{obs}	$\frac{k_{obs}(H)}{k_{obs}(D)}$	k_3	k_4
		s^{-1}		s^{-1}	
1.	CH_3-CH_2-CH_2-	10	–	3.5	6.5
2.	CH_3-CH_2-CD_2-	4	2.5	1.4	2.5
3.	CD_3-CD_2-CH_2-	0.7	14	0.27	0.45
4.	CD_3-CD_2-CD_2-	0.35	28	0.15	0.21
5.	CH_3-CH(D)-CH(D)- (*R*) (*R*)	(0.48)	(21)		

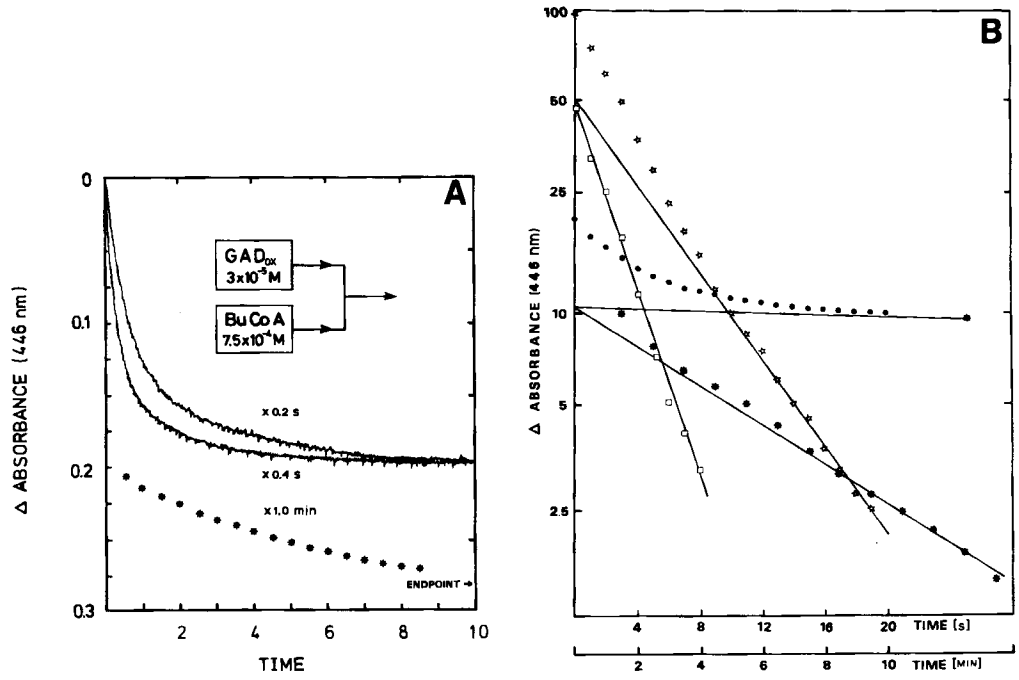


Fig. 3. *(A) Anaerobic half reaction of GAD with butyryl-CoA as measured in the stopped-flow instrument. (B) Semilogarithmic plot of the reaction course of GAD with perdeutero-butyryl-CoA.* (A) The enzyme and the substrate in 50 mM phosphate buffer, pH 7.6, were made anaerobic, and then reacted at 25°C. The lower part of the curve (★——★) was recorded with a conventional *x/t* recorder. (B) 0.03 mM GAD and 0.3 mM substrate were reacted under the conditions described in A. The slowest phase (★——★) was plotted first using the experimentally determined endpoint. The absorbance of this (third) phase was then subtracted from the residual part of the curve (●——●) to yield curve (☆——☆) (first and second phases). Repetition of the same procedure separates the first (□——□) phase. The minute time scale refers to the slowest phase (★——★). The vertical ΔA_{446} scale has arbitrary units

faster rates, the course of reduction is closely similar to and should be compared with that observed in the experiment depicted in Fig. 1. In particular, it shows three distinct phases of absorbance decrease at 450 nm. It should be pointed out that in all cases reported so far [5–8, 12, 23–25], the reductive half reaction of fatty-acyl-CoA dehydrogenase has been found to occur in two or three phases. The nature of the multiphasicity of the processes is not yet understood, although some hypotheses, like subunit nonequivalence, have been put forward [6, 8]. We would like to emphasize that in the present work only the minimal kinetic analysis necessary for the interpretation of the isotope effects will be attempted. A thorough analysis of the complex behaviour of GAD will be reported elsewhere (Schopfer, L., Ghisla, S., Massey, V. and Thorpe, C., unpublished). As also reported for the pig liver enzyme [6], we found that the ratio of absorbance changes occurring in the first two phases at 450 nm was ≈ 1 ($\pm 30\%$) and not dependent on the deuteration of substrate. We will focus in the present work on the rates and isotope effects observed in the first, fastest, reduction phase since this is the most accurately determined. The isotope effects found for the second phase are qualitatively similar. The rate ($t_{1/2} = 2-3$ min at

25°C) and extent (20–30% of total absorbance changes, cf. Fig. 6) of the third, slowest phase are essentially independent of the deuteration of substrate. This phase is much too slow to be catalytically significant. It is probably associated with conversions of oxidized substrate following dissociation from the reduced enzyme (cf. [8]) and might involve a (slow) endogenous hydratase activity of GAD as detailed elsewhere [26]. The extent of reduction observed during the three phases is strongly dependent on the substrate concentration, as will be detailed below (cf. Fig. 6), but is independent of deuteration.

The rates of the individual phases of flavin reduction were estimated either graphically, by successive subtraction of the slowest phase from the preceding phases in semi-logarithmic plots as shown in Fig. 3B, or by computer analysis using three sequential relaxation processes [7]. The agreement between the two methods was within 20–30%. As can be seen from the plot of the data in Fig. 4, the scatter of the data points is considerable (≈30%), in particular at low substrate concentrations where the spectral changes are small (cf. Fig. 6). This scatter is comparable to experimental results of others [6]. Fig. 4 shows that the observed rates (k_{obs}) are essentially independent of the concentration of substrate when the latter is >0.5 mM. The rates of the second phase behave similarly. Such a saturation behaviour has been predicted by Strickland et al. [27] for kinetic schemes in which substrate concentrations are all high enough to saturate the Michaelis complex, binding is a rapid-equilibrium process and when reduction is reversible, i.e. when the value of k_4 is finite. Such a scheme is represented in the framed part of Scheme 1. Clearly this scheme does not account for the biphasic (phases 1 and 2) behaviour (Schopfer, L., Ghisla, S., Massey, V. and Thorpe, C., unpublished). Since no acceptor is present ($k_5 = 0$), dissociation of product from its complex with reduced enzyme (k_7) can be assumed to be slower than the other relevant steps (k_1 to k_4). Product dissociation has been reported to be slow, $k \approx 0.01\ s^{-1}$, with the pig liver dehydrogenase [8] and thus is probably associated with the third slow phase also in the present case. In a plot such as that of Fig. 4, the extrapolation to the ordinate of the value of k_{obs} obtained at substrate saturation will yield $(k_3 + k_4)$, while extrapolation to the ordinate of the values obtained at low substrate concentrations should represent k_4 [27]. The plot of the rates observed with all substrates at low concentrations qualitatively follow this prediction.

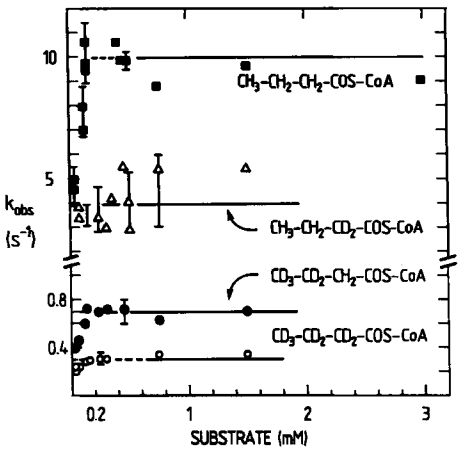


Fig. 4. *Dependence of the rate of the first reduction phase on the substrate concentration.* The single values are the average of two, three or four determinations obtained as shown in Fig. 3B from the primary data such as those of Fig. 3A. The substrates used were those shown in the figure. The bars indicate the representative scatter of data for selected points

However, only with the 'slowest' substrate, perdeutero-butyryl-CoA, does the quality of the primary results allow a satisfactory elaboration of the data according to the method of Strickland et al. [27]. This is shown in Fig. 5A, where the ordinate extrapolation of k_4 can be estimated as ≈0.21 (0.2–0.25) s^{-1}. A secondary plot of $(k_{obs} - k_4)/(1/[S])$ (Fig. 5B) yields $k_3 \approx 0.15\ s^{-1}$ and $K_d \approx 0.12$ mM. This indicates that the ratio $k_3/k_4 \approx 0.6$. If this ratio is applied to the case of normal non-deuterated substrate, where $(k_3 + k_4) \approx 10\ s^{-1}$, then $k_3 \approx 4\ s^{-1}$ and $k_4 \approx 6\ s^{-1}$. We have reported earlier [10] the experimental determination of the rate of 'reoxidation' of reduced GAD by the product crotonyl-CoA as 2.6 s^{-1}. Since this should be the lowest (limiting) value for k_4 it would indeed confirm the assumption that k_4 has values of the same magnitude as k_3. This would also be in agreement with the results of Murfin [5], who has deduced from competition experiments substantial rates for the 'reoxidation' of ox liver enzyme. We would like to state that, while these values should clearly be considered as mere estimations, they show a good coincidence with the ratios of $k_3/k_4 \approx 0.7$ estimated as detailed below.

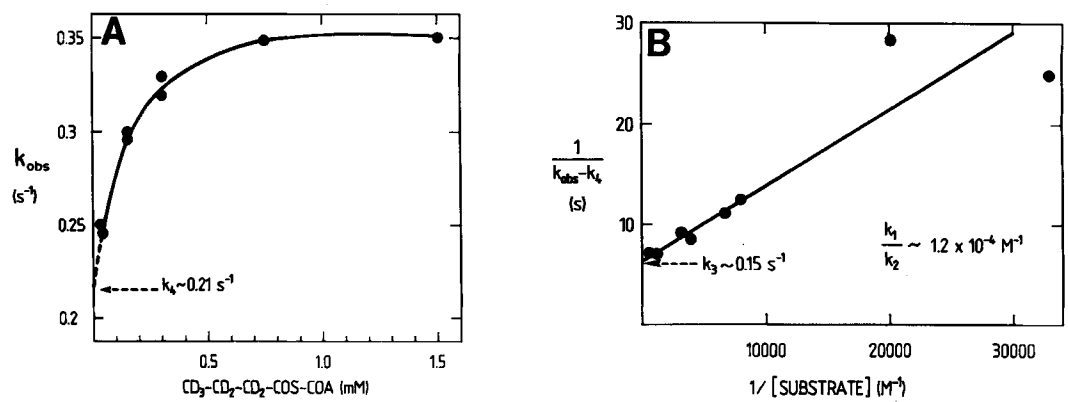


Fig. 5. *Determination of reaction parameters using perdeutero-butyryl-CoA.* (A) The data obtained for the first reaction phase of GAD reduction with perdeutero-butyryl-CoA from Fig. 4 were replotted on an expanded scale according to the method of Stickland et al. [27]. The extrapolated value for k_4 is ≈0.21 (0.03). (B) Double-reciprocal plot of $(k_{obs} - k_4)$ versus substrate concentration, and estimation of k_3 and K_d $(= k_2/k_1)$

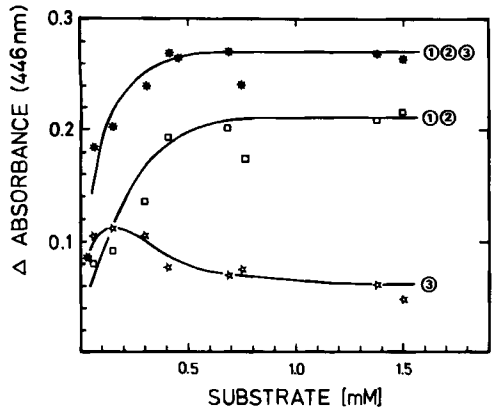


Fig. 6. *Dependence of extent of reaction of GAD on the concentration of butyryl-CoA derivatives.* The values shown are the average of the endpoints obtained using all substrates (i.e. non-deutero, α-deutero, β-deutero, and perdeutero-butyryl-CoA). Curve (★——★) shows the extent of reaction phases 1+2+3, curve (□——□) those of reaction phases 1+2 and (☆——☆) those of the third slowest phase

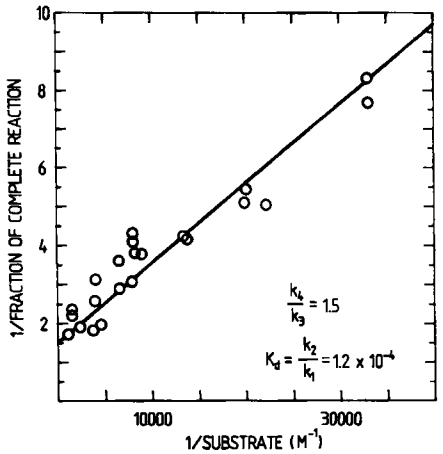


Fig. 7. *Double-reciprocal plot of the fraction of complete reaction versus substrate concentration.* The points represent the endpoint of the first two fast phases of enzyme reduction obtained as shown in Fig. 3B and using all substrates. Complete reduction was defined as the extent of absorbance decrease observed at 450 nm upon anaerobic titration with dithionite [12]. The line represents the theoretical values calculated for the endpoints of the reaction using the values shown for k_{1-4}

Dependence of extent of enzyme flavin reduction on substrate concentration

The extent of enzyme 'reduction', i.e. of the absorbance changes occurring in the 450-nm spectral region upon reaction with substrate shows a marked dependence on its concentration up to about 0.5 mM and appear to saturate at concentrations above 1 mM as shown in Fig. 6. The substrate concentration at 50% reaction is ≈0.1 mM; this thus probably reflects the magnitude of the primary binding step K_d. Most importantly, the extent of reduction is independent of the deuteration of substrate. That acyl-CoA dehydrogenases are 'bleached' to an extent which is dependent on the chemical constitution and, in particular, on the chain length of the substrate has been noted previously by several workers [12, 24, 28]. A scheme which might account for such a behaviour has been put forward already by Beinert and coworkers [1] and later by Thorpe et al. [12]; it essentially predicts that the extent of observed reduction at substrate saturation will reflect the internal equilibrium of steps k_3 and k_4 (Scheme 1). This would also be in keeping with the observation that the extent of enzyme flavin reduction depends on the redox potential of the flavin (at constant substrate concentration) [29]. In the extreme case of 5-deaza-FAD-GAD ($E_0 \approx -320$ mV) the reaction was found to proceed only in the 'reverse' direction [10]. In contrast to this Reinsch et al. [6], working with the pig liver enzyme, while observing only approximately 50% reduction using butyryl-CoA, asserted that the reaction was essentially irreversible (i.e. $k_4 = 0$). In order to obtain information on this specific controversial point, we have calculated the theoretical endpoint of the reaction for different substrate concentrations according to the method of Strickland [27], assuming values of K_d (k_2/k_1) = 0.12 mM and k_3/k_4 = 0.65. The results are compared in Fig. 7 with the experimentally obtained values, i.e. to the endpoint of the first- and second-phase reduction. Note that in this (thermodynamic) comparison the biphasic behaviour of the reduction course should not affect the validity of the conclusion since the relative extent of the first and second phases is approximately constant in the concentration range examined.

For this comparison, the extent of full reduction was defined by the absorbance at 450 nm observed upon reduction with octanoyl-CoA [12], which is similar to the degree of bleaching observed upon dithionite reduction. Since the same behaviour was observed with all substrates, regardless of the degree of deuteration, one important deduction follows: the isotope effect on the internal redox equilibrium (steps k_3/k_4, Scheme 1) must be close to 1, i.e. similar isotope effects apply to the step of 'reversal of reduction' as well as to reduction itself.

CONCLUSIONS

The main point emerging from the present study is the finding of an unusually large isotope effect during the dehydrogenation of acyl-CoA substrates. While the value of ≈2.5 for the isotope effect found with α-deutero-butyryl-CoA is within the normal range, and clearly is a primary effect, the value of ≈14 found with β-deutero substrate is unusually large and is close to the maximal value which might be expected for reactions not involving tunnelling [22]. The magnitude of the overall (i.e. protio/perdeutero-butyryl-CoA) isotope effect on k_{obs} ≈28, found for the first reduction phase (Table 2) is lower than the value of ≈50 reported by Reinsch et al. [6] and higher than those of ≈17 and ≈2 reported by Murfin [5] and Raichle [7], respectively, for the dehydrogenation of various α,β-tetradeuterated acyl-CoA substrates. It is likely that in all these instances the same mechanism is operative and that the differences in isotope effects result from difference in the protein/substrate interaction. The isotope effect of ≈1.3 observed by comparing the rates of perdeuterobutyryl-CoA (i.e. CD_3-CD_2-CD_2-COS-CoA) with (2*R*,3*R*)-(2,3-D_2)butyryl-CoA should be an approximation of the sum of the secondary isotope effects resulting from the presence of (nonreacting) deuterium at the positions 2, 3 and 4 of perdeutero-butyryl-CoA.

With respect to the mechanism of dehydrogenation, the comparison of the specific isotope effects (i.e. α or β) with the 'total' isotope effect (i.e. α + β) should yield clues to the reaction mechanism. In the case of a process involving definite intermediates, the isotope effects reflected by k_{obs} using α- and β-deuterobutyryl-CoA might behave either additively or reflect the slowest of the two steps. In contrast to this, in

a synchronous or concerted mechanism involving a single transition state a multiplicative effect would be expected. In the present case, the isotope effect of ≈28 is much closer (actually within the margin of error of the determination) to the 'multiplicative value' of 35 (i.e. 2.5×14) as compared to 16.5 (i.e. $2.5 + 14$). In our case the rupture of the α- and β-C-H bonds is thus likely to proceed with a high degree of concertedness. This interpretation is consistent with the large isotope effect of ≈50 found by Reinsch et al. [6], as well as with the results of Murfin [5]. It is also consistent with our interpretation [4, 10] that exchange of substrate α-H in butyryl-CoA occurs at the locus of reduced enzyme–enoyl-CoA complex and not at the level of a substrate α-carbanion. This interpretation does not exclude the possibility of α-hydrogen exchange with acyl-CoA derivatives in which 'the second step', i.e. the transfer of the β-hydride to the flavin is either hindered or impossible. In borderline cases exchange might be concurring with flavin reduction. The relatively small isotope effect found with α-deutero-butyryl-CoA is most probably not due to partial exchange of this hydrogen since the exchange rate is much slower than that of enzyme reduction [10]. The finding of a negligible equilibrium isotope effect is consistent with expectations [30] and also with the assumption that the α-hydrogen is not being exchanged during the time scale of our measurements. This observation is also consistent with the α-proton being abstracted by a monofunctional base. In fact, Fendrich et al. [31] have identified a glutamic acid carboxylate at the active centre of butyryl-CoA dehydrogenase from *Megasphaera elsdenii* and have proposed that it might work in α-hydrogen abstraction.

From the spectroscopic experiments of Fig. 1, it can safely be concluded that during reduction of pig kidney GAD with butyryl-CoA to form the complex of reduced-enzyme–crotonyl-CoA no intermediates are observable except for the Michaelis complex. Our results are compatible with a freely reversible redox system, the observed ratio of oxidized and reduced enzyme species obtained in the presence of substrate reflecting the position of the internal redox equilibrium of the steps k_3 and k_4 (Scheme 1). This is in contrast to the interpretations of others [6], while it supports the original concept of Beinert [1, 21, 23] as extended by Thorpe et al. [12] and is also in agreement with the general layout of Murfin [5]. In particular we have found no evidence for intermediates such as those proposed by Ikeda et al. [9] in which a hydride is supposed to be 'shared' between oxidized flavin and substrate. The interpretation of Reinsch et al. (irreversible reaction) [6] might have relied on their supposedly observed lack of reaction of reduced enzyme with crotonyl-CoA. It is conceivable that their results [6] have been affected by the relative instability of this unsaturated substrate, especially in view of our findings that crotonase (hydratase) activities can be present in acyl-CoA dehydrogenase preparations [26].

Comparison of our results with similar studies by others [5–9, 17] unveils substantial differences in the kinetic behaviour among acyl-CoA dehydrogenases from different organs or organisms and suggests structural diversities. On the other hand, several common properties also emerge, like the biphasic reduction course and attainment of a redox equilibrium. At present only a scheme which incompletely describes the kinetic behaviour of general acyl-CoA dehydrogenase can be presented (Scheme 1); in particular, it can only partially account for the multiphasic behaviour of the events observed during the rapid kinetics measurements. Steps k_{7-11} will occur subsequently to anaerobic reduction, they represent dissociation of product and attainment of a thermodynamic equilibrium involving all species shown. This equilibration is likely to be connected to the third slow kinetic phase; it will, however, be more complicated than shown since product is also transformed, by the endogenous hydratase activity described elsewhere [26], to β-hydroxy-butyryl-CoA and since the latter also will bind to oxidized and reduced GAD.

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