Fatty Acyl-Coenzyme A Dehydrogenases

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The general enzymology of β-oxidation has suffered extraordinary neglect since the broad outlines of the pathway were established in the 1950's. For the acyl-CoA dehydrogenases the contributions from the Institute for Enzyme Research at the University of Wisconsin still represent the major part of our knowledge. This group of flavoprotein enzymes introduces the trans-α,β olefinic bond in the hydrocarbon chain of a fatty acyl thioester, making possible the subsequent hydration and further oxidation at the β-position. Some of the unusual properties of these enzymes have impeded their study in the past, but these very properties are essential to an understanding of the mechanism and control of fatty acid oxidation. In the present paper some of the important unanswered questions are highlighted.

Chain-length specificity

In mammalian systems there are three fatty acyl-CoA dehydrogenases differing, but overlapping, in chain-length specificity. In the past their similarity has hindered purification and led to low yields. With improved procedures, however, this problem has now been overcome (Murfin, 1974; Hall & Kamin, 1975; Hall et al., 1976). Why are three different enzymes necessary to catalyse what is, in chemical terms, essentially the same reaction? The obvious answer is that the different physical properties of long- and short-chain substrates dictate different properties for their binding sites. If so, however, one must explain the apparent absence of corresponding multiple forms of the β-hydroxyacyl-CoA dehydrogenases from mammals. Possibly only enzymes that catalyse rate-limiting reactions have multiple forms. Different steps in the β-oxidative sequence may, of course, be rate-limiting for substrates of different chain length. Genetic evidence suggests, however, that in Escherichia coli, there are two o xoacyl-CoA thiolases, two enoyl-CoA hydratases and two β-hydroxyacyl-CoA dehydrogenases (Klein et al., 1971). Purification studies have so far failed to substantiate this claim (Binstock et al., 1977).

Acceptor specificity

Most flavoproteins pass on substrate-donated reducing equivalents either to oxygen or to such other general electron acceptors as cytochrome c and nicotinamide nucleotides. The mammalian acyl-CoA dehydrogenases, however, when reduced by substrate, are stable to oxygen and react only slowly with most electron-accepting dyes (Beinert, 1963a). Phenazine methosulphate and Meldolablau are the best artificial acceptors so far discovered, and both have been used to couple transfer of reducing equivalents from acyl-CoA dehydrogenases to cytochrome c, 2,6-dichlorophenol-indophenol or tetrazolium dyes. Meldolablau has the advantage over phenazine methosulphate that it is stable to light and is not autoxidisable (Dommes & Kunau, 1976).
The natural acceptor for the acyl-CoA dehydrogenase is another flavoprotein, the 'electron transferring flavoprotein' ('ETF'), ET flavoprotein (Beinert, 1963b), which serves the same function for sarcosine and dimethylglycine dehydrogenases (Beinert & Frisell, 1962; Hoskins, 1966), and feeds reducing equivalents to the respiratory chain.

The requirement of the acyl-CoA dehydrogenases for ET flavoprotein, and their contamination with it, have proved considerable obstacles to the assay of catalytic activity in the past. In addition to this practical problem, there is the more theoretical question as to why one flavoprotein should require another flavoprotein to transfer its reducing equivalents to the respiratory chain. The most satisfactory explanation remains that offered by Beinert & Crane (1956), namely that ET flavoprotein, by serving as an 'adaptor' for several dehydrogenases, relieves potential congestion in the vicinity of the respiratory-chain assembly.

The transfer of electrons from flavoproteins to the respiratory chain usually involves the mediation of Fe–S proteins. Succinate dehydrogenase, which catalyses a desaturation reaction analogous to that of the acyl-CoA dehydrogenases, possesses Fe–S clusters in addition to flavin. Despite an early claim (Mahler, 1954) that butyryl-CoA dehydrogenase has a copper prosthetic group, the acyl-CoA dehydrogenases and ET flavoprotein appear to be devoid of functional metal ions (Steyn-Parv & Beinert, 1958a; Beinert, 1963a,b), and until recently their mode of coupling to the respiratory chain remained obscure. An Fe–S-containing flavoprotein, initially identified by its e.p.r. signal at $g = 2.086$ in the reduced state (Centre 5; Ohnishi et al., 1972), has now been purified from ox heart mitochondria and shown to serve as a specific ET-flavoprotein dehydrogenase (Ruzicka & Beinert, 1975). This protein is bound to the inner mitochondrial membrane and equilibrates with the NADH and succinate dehydrogenase systems (Ohniski et al., 1972). It is especially prominent in the e.p.r. spectrum of mitochondria from brown adipose tissue, which is adapted for fatty acid oxidation (Flatmark et al., 1976).

There are thus three flavoproteins in the electron-transport sequence linking acyl-CoA substrates with the respiratory chain. It is not yet known in what molar ratios these proteins are present in vivo, nor whether any or all of them are associated to form stable functional complexes, The acyl-CoA dehydrogenases and ET flavoprotein are readily obtained separate from one another in free solution. On the other hand, dependence on protein diffusion would severely limit the maximum rate of turnover in vivo. If all the protein elements are associated, then presumably some respiratory-chain assemblies must be associated with short-chain acyl-CoA dehydrogenase, some with medium-chain, and some with long-chain, since all three utilize ET flavoprotein. There is certainly no evidence of intermediary electron carriers of low molecular weight, although Hall & Kamin (1975) and Hall et al. (1976) report that an unidentified factor improves coupling between acyl-CoA dehydrogenases and ET flavoprotein.

Of the non-mammalian acyl-CoA dehydrogenases, the short-chain enzyme of *Megasphaera (= Peptostreptococcus elsdenii* has been studied in most detail (Engel & Massey, 1971a,b; Engel, 1972). In this case an ET-flavoprotein transfers reducing equivalents from NADH and $\alpha$-lactate dehydrogenase to the acyl-CoA dehydrogenase, which functions as an enoyl-CoA reductase (Baldwin & Milligan, 1964; Whitfield & Mayhew, 1974; Brockman & Wood, 1975).

The mitochondrial $\beta$-oxidation system in plants has not been extensively investigated. A separate system appears to operate in the glyoxysomes of fat-storing plants, primarily geared to the provision of C$_2$ units for biosynthesis rather than energy production. The acyl-CoA dehydrogenase in this system is thought to use oxygen as its electron acceptor, but it has not been purified (Hutton & Stumpf, 1969; Cooper & Beevers, 1969).

**Tight binding of substrates**

One of the most striking properties of the acyl-CoA dehydrogenases is their very high affinity for substrates and products. Steyn-Parv & Beinert (1958a) found that 1 mol of added radioactive substrate per mol of enzyme-bound FAD remained bound
to pig acyl-CoA dehydrogenases after dialysis, (NH₄)₂SO₄ precipitation, charcoal treatment, Dowex-2 treatment, ultracentrifugation or electrophoresis. The bound substrate was released under denaturing conditions and could also be rapidly displaced from native enzyme by an excess of unlabelled substrate. Beinert (1963a) suggests that the dissociation constants of these tight complexes are less than 100 pM. This estimate is not based on quantitative measurements of free and bound thioester, however. Dissociation constants for the tight complexes formed by the short-chain acyl-CoA dehydrogenase of *Megasphaera elsdenii* with pent-2-enoyl-CoA and acetoacetyl-CoA, measured by spectrophotometric titration, are approximately 1 μM (P. C. Engel, unpublished work).

The dissociation constant for any binary enzyme-substrate complex compulsorily involved in the catalytic cycle imposes a limit on the maximum rate of turnover. If, for example, the dissociation constant for an enzyme-product complex is 10 nm and one assumes an upper limit of 5 × 10⁸ s⁻¹ M⁻¹ for the second-order 'on' rate constant for formation of the complex, then the corresponding upper limit for the 'off' rate constant is 5 s⁻¹. The turnover rate cannot exceed the maximum rate of product release. Quoted turnover numbers for the acyl-CoA dehydrogenases (e.g. Beinert, 1963a; Engel & Massey 1971a; Hall & Kamin, 1975) are in fact of the order of 1–10 s⁻¹. An enzyme-product complex with a dissociation constant of 100 pM could not therefore be on the catalytic route. The dissociation constant may be influenced, however, by the presence of the electron acceptor. It is not yet known definitely whether the reduced enzyme is reoxidized before the oxidized acyl-CoA product leaves and, if so, whether the reduced acceptor leaves the enzyme surface before or after the acyl-CoA product.

What is the significance of the tight binding of substrates by the acyl-CoA dehydrogenases? The total pool of CoA, as thiol or thioester, is small, and the concentration of any single acyl-CoA is inevitably much smaller. This may present little difficulty if all the enzymes of β-oxidation form a multienzyme complex in which each enzyme feeds substrate to the next, but there is no evidence that the acyl-CoA dehydrogenases form part of such a complex. In *E. coli* the other β-oxidative enzymes are associated with a single polypeptide chain, but this multifunctional enzyme lacks acyl-CoA dehydrogenase activity (Binstock et al., 1977). In searching for free intermediates of β-oxidation in a preparation derived from rat liver mitochondria, Stanley & Tubbs (1974) found predominantly saturated acyl-CoA. In cell extracts, measured specific activities of the acyl-CoA dehydrogenases are much lower than those of the other enzymes of β-oxidation (e.g. Bunyan & Greenbaum, 1965; Weeks et al., 1968). It thus seems that the acyl-CoA dehydrogenase reactions may be rate-limiting and may depend on diffusion of substrates that are present at very low concentrations. If so, a high affinity of the enzymes for their substrates may be indispensable.

Another important aspect of the tight binding of acyl-CoA compounds by acyl-CoA dehydrogenases is the opportunity that it appears to provide for tight physiological and pharmacological control. In the study of the control of fatty acid oxidation there has been an emphasis on the supply of fatty acid chains. In view of the scarcity of CoA, it is difficult to believe that there is not also direct control via the concentrations of acetyl-CoA and acetoacetyl-CoA. The properties of the acyl-CoA dehydrogenases are ideally tailored to provide such control. There is no need to postulate allosteric inhibition, because the CoA moiety common to all β-oxidation intermediates facilitates simple competitive inhibition by the end products of the pathway. The CoA moiety contributes a large part of the binding affinity (Engel, 1972), and one may predict, therefore, that acetyl-CoA and acetoacetyl-CoA are likely to be effective inhibitors, especially of the short-chain acyl-CoA dehydrogenase. This has been shown for acetoacetyl-CoA with the enzyme from *Megasphaera elsdenii* (Engel & Massey, 1971b), but not with the mammalian enzyme. The sensitivity of mammalian acyl-CoA dehydrogenases to possible physiological inhibitors should be explored.

The short-chain dehydrogenase, as normally isolated, is green. This colour, attributed initially to copper (Mahler, 1954) and then to a flavin-protein interaction (Steyn-Parvé & Beinert, 1958a), has now been shown to be due to the presence of tightly bound
acyl-CoA (Engel & Massey, 1971b) removable by acid precipitation of the protein. Coloured charge-transfer complexes (see Abramovitz & Massey, 1976) can be produced with various acyl-CoA ligands (Engel & Massey, 1971b; Engel, 1972), all with either \(\alpha,\beta\)-unsaturated or \(\beta\)-oxo aliphatic or alicyclic acyl chains. The natural ligand remains unidentified, however. The partly reversible decline of 're-greening' capacity in the acid extract (Engel, 1972) suggests that this ligand can undergo a reversible rearrangement, perhaps a cyclization analogous to the formation of triacetate lactone and CoA from triacetyl-CoA by fatty acid synthetase (Nixon et al., 1968). This view is now supported by detailed studies (Engel & Jones, 1978) of the de-greening of the green enzyme by mercurial, which appears to be due to reaction of the ligand rather than the protein. This reaction is immediately reversible with thiols in the short term. Over several hours, however, there is a pH-dependent decline in the extent of this immediate reversibility, and, as with the acid extract, this decline is itself in part slowly reversible. These studies should provide useful clues to guide future attempts to identify the bound acyl-CoA, which may well prove to be a significant natural inhibitor.

A third important aspect of tight substrate binding is its effect on the oxygen reactivity of the flavoprotein. A purified mammalian acyl-CoA dehydrogenase, reduced with dithionite, is readily reoxidized by oxygen. Substrate-reduced enzyme, however, remains reduced in the presence of oxygen for hours (Beinert, 1963a). In this state the enzyme contains tightly bound acyl-CoA product and displays a broad long-wave-length absorption band (500–600 nm), originally attributed to the semiquinoid half-reduced form of the flavoprotein (Beinert, 1956). The e.p.r. signal expected for a free radical is absent, however, (Beinert & Sands, 1961), and it now appears that complexes of this type involve charge transfer between reduced flavin as donor and oxidized product as acceptor (Murfis, 1974; Abramovitz & Massey, 1976).

In the presence of ET flavoprotein, substrate-reduced enzyme is readily reoxidized. Since the acyl-CoA dehydrogenases are readily dissociable from ET-flavoprotein, their striking stability to oxygen when they are reduced with substrate may perhaps be an adaptation to ensure safe delivery of the reducing equivalents to the respiratory chain in an aerobic cell. The short-chain acyl-CoA dehydrogenase of \(\text{Megasphaera elsdenii}\), by contrast, is rapidly reoxidized by oxygen even after reduction by substrate. Since this enzyme, from an anaerobe, normally functions in the direction of substrate reduction, it clearly needs no protection against oxygen.

Much remains to be learnt about this important group of enzymes. It is to be hoped that the availability of improved methods of assay and purification will stimulate greater activity in this area.

Beinert, H. (1963a) Enzymes 2nd Ed. 7, 447–466
Beinert, H. (1963b) Enzymes 2nd Ed. 7, 467–476
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3-Oxoacyl-Coenzyme A Thiolases and L-3-Hydroxyacyl-Coenzyme A Dehydrogenases

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L-3-Hydroxyacyl-CoA dehydrogenase and 3-oxoacyl-CoA thiolase catalyse the two terminal steps of the β-oxidation spiral. In addition to their role in the oxidation of triacylglycerols derived fatty acids, both these steps occur in the oxidation of the cholesterol side chain, the oxidation of the carbon skeleton of isoleucine and in the malonyl-CoA-independent mitochondrial elongation of long-chain fatty acids. The 3-oxoacyl-CoA thiolase reaction is also involved in both the synthesis and degradation of ketone bodies and as the first committed step in sterol biosynthesis from acetyl-CoA. L-3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) is a NAD-linked mitochondrial enzyme of widespread tissue distribution. Originally first purified to homogeneity by Stern (1957), Noyes et al. (1974) described an improved purification from pig heart and have made an extensive study of the enzyme. With the exception of an opposite stereospecificity of H-transfer from NADH, this enzyme shows similarities to the mitochondrial L-malate dehydrogenase in molecular weight (67000), subunit number (2, identical), amino acid composition, pI of 8.95 and susceptibility to inhibition by the keto substrate. The two enzymes also cross-react immunologically to a significant extent. The kinetic mechanism with acetoacetyl-CoA as substrate has been shown by Schifferdecker & Schulz (1974) to be of the compulsory ordered type (like that of malate dehydrogenase). The highly inhibitory effect of acetoacetyl-CoA on both directions of the reaction prompted the above authors to propose that β-oxidation could be regulated at this step by changes in acetoacetyl-CoA concentration. This must remain conjecture until mitochondrial acetoacetyl-CoA concentrations have been determined.

Although L-3-hydroxyacyl-CoA dehydrogenase from pig heart shows activity towards a range of 3-oxoacyl-CoA substrates, this activity is highest with acetoacetyl-CoA (B. Middleton, unpublished work) and falls off with increasing chain length of the acyl group. The enzyme is virtually inactive towards branched-chain oxoacyl-CoA compounds. Since for all other steps of β-oxidation there exist long-chain and short-chain acyl-CoA-specific enzymes, it seems unlikely that the hydroxyacyl-CoA dehydrogenase reaction would not conform in this respect. Further, from their studies on Escherichia coli mutants, Klein et al. (1971) have suggested that in this organism, at least, there exist two or more types of enoyl-CoA hydratase, 3-hydroxyacyl-CoA