Structure of the cubic core and the lipoamide dehydrogenase component of the pyruvate dehydrogenase complex.

Mattevi, Andrea

SUMMARY

The pyruvate dehydrogenase multienzyme complex (PDH)[ref.1] catalyzes the oxidative decarboxylation of pyruvate coupled to the synthesis of acetylCoA and to the reduction of a molecule of NAD^+. This is a key reaction in the metabolism of glucose, because it occurs at the junction of glycolysis and the Krebs cycle. PDH is a member of the family of the oxoacid dehydrogenase multienzyme complexes, which, in addition to PDH, also comprises the 2-oxoglutarate dehydrogenase (OGDH) and the branched-chain oxoacid dehydrogenase (BCDH). These complexes are large systems having a molecular weight of 5-10 million daltons and comprising multiple copies of no less than three different enzymes. The decarboxylase component (E1), a thiamin pyrophosphate dependent protein, carries out the decarboxylation of the oxoacid and attaches the acyl group to lipoamide. The transacylase enzyme E2 then transfers the acyl moiety to CoA, releasing lipoamide in the reduced state. The latter is reoxidized by the FAD dependent enzyme lipoamide dehydrogenase (E3), which transfers the reducing equivalents to NAD^+. Specific components of PDH are pyruvate decarboxylase (E1p) and dihydrolipoyl transacetylase (E2p), whereas the corresponding enzymes of OGDH are oxoglutarate dehydrogenase (E1o) and dihydrolipoyl transsuccinylase (E2o). Also BCDH has its own specific E1b and E2b enzymes. Thus, E3 is the only component common to all these multienzyme systems.

In all oxoacid dehydrogenases E2 plays a key functional and structural role. Namely, E2 carries the lipoamide cofactor, which is covalently bound to the ε-amino group of a lysine side chain. Moreover, E2 forms the inner core of the complexes to which the peripheral members E1 and E3 are bound. Consistent with this essential role, E2 has a unique modular arrangement[ref.2,3], comprising three types of domains: (i) the lipoyl domain about 100 residues long, bears the covalently bound lipoamide; (ii) the E1/E3 binding domain comprising 50 residues, is essential for the association of the peripheral subunits; and, (iii) the catalytic domain of approximately 250 residues, possesses the active site and forms the inner highly symmetrical[ref.4] core of the complexes. The domains are connected to each other by highly flexible polypeptide regions, which are essential for the functioning of the systems. Thanks to the linkers, the lipoyl domains form a "super-arm", which allows the lipoamide cofactor to visit the catalytic centers of the E1, E2 and E3 enzymes.

In this thesis, the crystal structures of the E3 component and of the catalytic domain of E2p are presented. These are the first atomic structures of the cubic core and of the E3 component of the pyruvate dehydrogenase and related multienzyme complexes. The structural features of the two enzymes are analyzed in the context of their catalytic functioning and of their participation to the overall activity of the oxoacid dehydrogenases.

Lipoamide dehydrogenase (E3)

The crystal structure of *Azotobacter vinelandii* lipoamide dehydrogenase was initially solved by Schierbeek et al.[ref.5] using a combination of isomorphous and molecular replacement. The

results of the crystallographic refinement of this 100,000 daltons dimeric flavoenzyme are presented in chapter 2. The final model has an R-factor of 19.8% for 44,292 reflections at 2.2 Å resolution. Each monomer of the E3 dimer comprises four domains and the active site is located at the interface between the two subunits. The unique combination of the flavin ring with an adjacent disulphide bridge generates the redox center involved in the electron transfer reaction between the substrates, lipoamide and NAD^+. The flavin ring separates the NAD^+ and the lipoamide binding sites, positioned on the two opposite faces of the reactive ring of FAD. The overall structure of the E3 appears to be similar to that of human glutathione reductase[ref.6], in agreement with the 26% sequence identity. Nevertheless, a comparison between the two enzymes reveals a number of differences in their tertiary and quaternary structure. In each subunit of lipoamide dehydrogenase the NAD-binding domain and the interface domain appear to be differently oriented with respect to the FAD-binding domain by 7.1° and 7.8°, respectively. Furthermore, the two subunits of E3 are shifted with respect to each other by more than 4 Å, when their mutual position is compared with that observed in glutathione reductase. Remarkably, in spite of these modifications the catalytic centers remain virtually identical in the two enzymes.

The crystal structure of the complex between *Pseudomonas putida* E3 and the substrate NAD^+ is presented in chapter 3. The structure was solved by molecular replacement and refined at 2.45 Å resolution to a final crystallographic R-factor of 21.3%. In this crystal form *P. putida* E3 contains one NAD^+ molecule per subunit. The adenine-ribose moiety of the substrate occupies an analogous position as in the structure of glutathione reductase, whereas the nicotinamide-ribose moiety is far removed from its expected position near the isoalloxazine ring and points into solution. This suggests a dependence of the substrate binding on the redox state of the protein. Furthermore, the structure of *P. putida* E3 reveals the conformation of the C-terminal residues which fold 'back' into the putative lipoamide binding region. The C-terminus has been proven to be important for activity by site directed mutagenesis. However, the distance of the C-terminus to the catalytically essential residues is still surprisingly large, over 6 Å, and the precise role of the C-terminus still needs to be elucidated.

Pseudomonas fluorescens lipoamide dehydrogenase has a high 84% sequence identity to the same protein from *A. vinelandii*. However, the two enzymes present significant differences in their thermostability and redox properties. The melting temperature of *P. fluorescens* E3 is 6° higher than that of the *A. vinelandii* enzyme. Moreover, unlike the latter, *P. fluorescens* E3 cannot be reduced to the EH_4 state, in which both the flavin ring and the catalytic disulphide are completely reduced ($FADH_2$.SH.SH)[ref.7]. In order to obtain some clues about the causes of these differences between the two proteins, the structure of *P. fluorescens* E3 was solved by molecular replacement and refined to an R-factor of 19.4% at 2.8 Å resolution. As expected, the structure of *P. fluorescens* E3 is closely related to that of the same flavoprotein from *Azotobacter vinelandii* with an r.m.s. difference of 0.53 Å for 932 Cα atoms. The residues in the active site are identical, while 89% of the interface residues are

the same in the two proteins. Thus, only few structural variations are responsible for the differences in thermostability and redox properties between the two homologous proteins. Among them, in the *A. vinelandii* molecule a threonine to alanine (T452A) mutation leaves a buried carbonyl oxygen, located at the subunit interface and in proximity of the flavin ring, unpaired to any H-bond donor. This might account for the greater stability of *P. fluorescens* E3 with respect to the *A. vinelandii* enzyme.

The catalytic domain of dihydrolipoyl transacetylase (E2p)

The catalytic domain of *A. vinelandii* E2p (residues 386-637) forms the core of the pyruvate dehydrogenase, by assembling 24 subunits into oligomers, having octahedral 432 symmetry. As described in chapters 5 and 6, the structure of *A. vinelandii* E2pCD was solved by multiple isomorphous replacement and refined to a crystallographic R-factor of 18.7% at 2.6 Å resolution. The 24 subunits appear to be arranged in a hollow cubic structure with edges of 120 Å and pores of a diameter of 30 Å crossing each face of the cube. The true building block is the E2p trimer, eight of which occupy the corners of the cube. The trimer is stabilized by extensive intermolecular interactions between threefold related subunits, whereas the interactions along the twofold axis leading to the assembly of the trimers into the cubic 24-mer involve only a few C-terminal residues, with four inter-subunit hydrogen bonds. The topology of each catalytic domain subunit is similar to chloramphenicol acetyltransferase (CAT), comprising a central β sheet surrounded by five α helices. The sequence identity between the two enzymes is only 17%. A remarkable difference between E2pCD and CAT is given by the N-terminal residues. In CAT, these fold back onto the carboxyl-terminal remainder of the subunit, while in E2pCD they form an arm which interacts with a neighboring subunit, thus lacing the subunits of the trimer, together.

The catalytic center of E2pCD is located at the interface between two threefold related subunits and consists of a 30 Å long channel, whose two entrances form the CoA and lipoamide binding sites. In the 24-meric cube, the trimers are oriented such that CoA must enter its binding site from the inside of the cube, whereas lipoamide enters from the outside. In this way, the flexible lipoyl domains on the surface of the cubic core will allow the covalently bound lipoamide to visit the active sites of E2 and of the peripheral subunits E1 and E3. In chapter 5, a model for the entire cubic-octahedral PDH is presented, based on the crystal structures of E2pCD and E3, combined to the wealth of electron microscopy and mutagenesis data.

The substrate binding and the catalytic functioning in E2p is analyzed in chapter 7, where the structure of six complexes and derivatives of *A. vinelandii* E2pCD are described. The binary complexes of the enzyme with CoA and lipoamide were determined at 2.6 Å and 3.0 Å, respectively. The two substrates are bound in an extended conformation, at the two opposite entrances of the 30 Å long catalytic channel. The reactive thiol groups of both substrates are in hydrogen-bond distance from the side chain of His 610, supporting the indication, derived from the similarity with chloramphenicol acetyl transferase, that this histidine side chain acts

as general base catalyst in the deprotonation of the reactive thiol of CoA. The deprotonation is the first step of the reaction catalyzed by E2pCD, which is thought to proceed via a negatively charged tetrahedral intermediate. The binary complex between E2pCD and hydrogen sulphite supports this hypothesis, showing that the anion is bound in the middle of the catalytic center, which is therefore capable of hosting and stabilizing a negative charge. The structure of the binary complex with hydrogen sulphite further suggests that transition state stabilization can be provided by a direct hydrogen-bond between the side chain of Ser 558 and the oxyanion of the putative intermediate. Quite unexpectedly, the structure of a ternary complex in which CoA and $Lip(SH)_2$ are simultaneously bound to E2pCD, reveals that CoA has an alternative, non-productive, binding mode. In the abortive ternary complex, CoA adopts a helical conformation with two intramolecular hydrogen bonds and the reactive sulphur of the pantetheine arm positioned 12 Å away from the active site residues involved in the transferase reaction.

The structures of the binary and ternary complexes between E2pCD and its substrates offer a clue for understanding the different substrate specificities of the various E2 transacylases forming the core of oxoacid dehydrogenase multienzyme complexes. The investigation of the three-dimensional structure of E2pCD combined with the analysis of the amino acid sequences suggests that the nature and size of only a few side chains part of the catalytic center might be the major determinants for tuning the substrate specificity among the various E2 transacylases.

1. Reed, L.J. (1974). Acc. Chem. Res., 7, 40-46.
2. Perham, R.N. (1991) Biochemistry, 30, 8501-8512.
3. Robien A. et al. (1992). Biochemistry, 31, 3463-3471; Dardel F. et al. (1991), Eur. J. Biochem., 201, 203-209.
4. Oliver, R.M. & Reed, L.J. (1982). In "Electron microscopy of proteins" (Harris, J.R. ed.), 2, 1-48, Academic Press, London.
5. Schierbeek A.J. et al. (1989), J. Mol. Biol., 206, 365-379.
6. Karplus, P.A. & Schulz, G.E. (1987), J. Mol. Biol., 195, 701-729.
7. Benen, J.A.E. (1992). Ph.D. Thesis, University of Wageningen (The Netherlands).