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Residues in the acetyl CoA binding site of pyruvate carboxylase involved in allosteric regulation

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ABSTRACT

We have examined the roles of Asp1018, Glu1027, Arg469 and Asp471 in the allosteric domain of *Rhizobium etli* pyruvate carboxylase. Arg469 and Asp471 interact directly with the allosteric activator acetyl coenzyme A (acetyl CoA) and the R469S and R469K mutants showed increased enzymic activity in the presence and absence of acetyl CoA, whilst the D471A mutant exhibited no acetyl CoA-activation. E1027A, E1027R and D1018A mutants had increased activity in the absence of acetyl CoA, but not in its presence. These results suggest that most of these residues impose restrictions on the structure and/or dynamics of the enzyme to affect activity.

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1. Introduction

Pyruvate carboxylase [EC 6.4.1.1] is a biotin-dependent enzyme that supplies oxaloacetate for replenishment of tricarboxylic acid cycle intermediates and in some specialised mammalian tissues, for gluconeogenesis, glucose-induced insulin release and neurotransmitter synthesis [1,2]. The enzyme from most organisms is highly regulated by the allosteric activator, acetyl CoA although there is some variation of the dependence of the enzyme on acetyl CoA, in terms of its activity in the absence of acetyl CoA and the K_a for activation [3,4]. In addition, the cooperativity of the activation of PC by acetyl CoA varies between species [3], with the Hill coefficient for the activation of the *Rhizobium etli* enzyme (RePC) being about 2.8 [5]. In recent years, the structures of the α_4 tetrameric pyruvate carboxylase holoenzyme from *R. etli*, *Staphylococcus aureus* (SaPC) and *Listeria monocytogenes* (LmPC) have been solved [6–10] and the catalytic mechanism of the RePC has been well characterised [11–15]. However, many aspects of the mechanism of action of acetyl CoA in its role as an allosteric activator of RePC remain to be established.

Fig. 1 shows the structure of the RePC monomer and the reactions occurring in the biotin carboxylase (BC) and carboxyltransferase (CT) domains, with the biotin being covalently attached to the mobile biotin carboxyl carrier protein (BCCP) domain. The allosteric domain, where acetyl CoA binds, occupies a central position between the three other domains [7]. The RePC tetramer is very asymmetrical and only two of the four subunits had ethyl CoA (an analogue of acetyl CoA) bound to them. Only this pair of
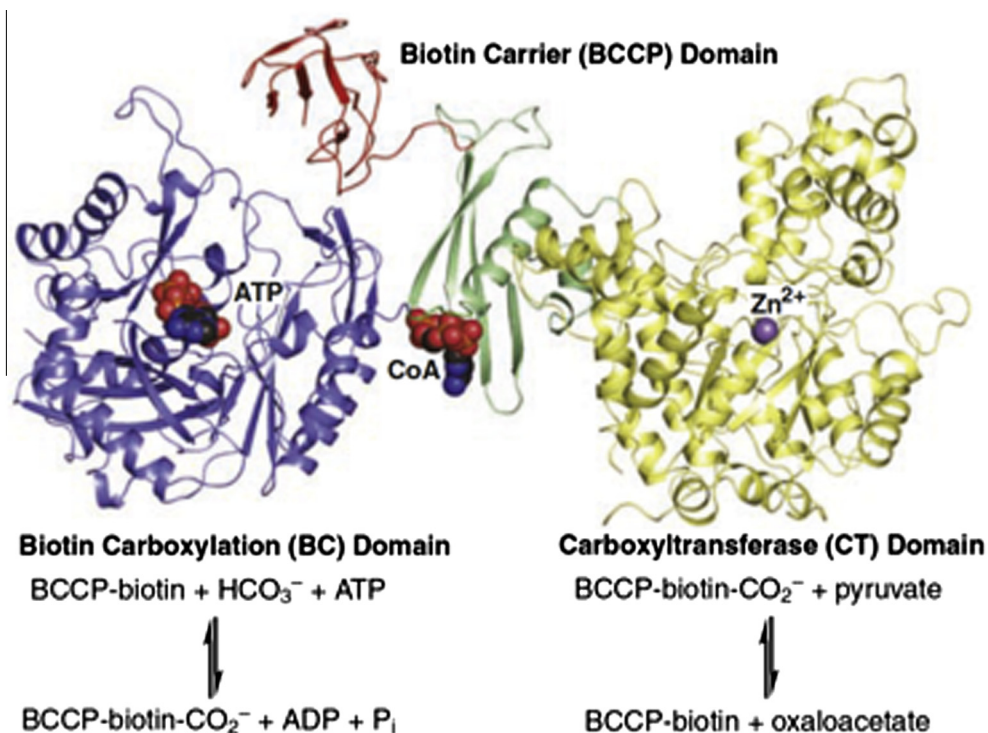
Fig. 1. The domain structure of the RePC subunit and the reactions catalysed by the BC and CT domains. Different colours in the primary structure of the enzyme represent various portions of the polypeptide attributed to the biotin carboxylase (BC), carboxyltransferase (CT), biotin carboxyl carrier protein (BCCP) and allosteric domains [6]. Reproduced with permission from [7].

subunits, on one face of the tetramer, appeared to be correctly con-figured to carry out the inter-subunit catalysis demonstrated to occur whereby the biotin bound to one subunit is carboxylated in its own BC domain but transfers its carboxyl group to pyruvate in its partner's CT domain [7]. The main locus of action of acetyl CoA is in the BC domain [3]. Fig. 2 shows part of the allosteric domain, with residues that directly interact with acetyl CoA and those that interact with these residues. Arg427 and Arg472 were the target of earlier mutagenesis studies that showed their crucial role in acetyl CoA binding and activation of RePC [5]. The current work extends these studies to examine the roles in the allosteric action of acetyl CoA of Arg469 and Asp471 residues, which interact directly with bound acetyl CoA, and Glu1027 which interacts with Arg472, when acetyl CoA is bound. In addition, the role of Asp1018 was examined, since this residue was shown to interact with Arg427 in the absence of acetyl CoA, but not in its presence [7].

2. Materials and methods

2.1. Construction of mutants

Mutations of Arg469 and Asp471 residues were performed on a 1.4 kb SacII–XhoI DNA fragment of R. etli PC gene, encompassing the BC domain while those of Asp1018 and Glu1027 residues were performed on a 1.0 kb BamHI–NotI fragment of R. etli PC gene, encompassing the BCCP domain. Mutagenesis was performed using a site-directed mutagenesis kit (Agilent) as described previously [7,11] (see Table 1 for the mutagenic primers used). The putative mutagenic clones were verified by automated DNA sequencing (Macrogen). Appropriate fragments of the correctly mutagenized clones were excised with SacII–XhoI or BamHI–NotI and used to replace the equivalent wild-type fragments in the full length RePC gene in pET17b vector [6].

2.2. Preparation of WT and mutant RePC

pET17b plasmid containing wild-type RePC or mutants was co-transformed with pCY216 plasmid encoding Escherichia coli bio-tin protein ligase (BirA) into E. coli BL21 (DE3). RePC was expressed and purified as described previously [11].

2.3. Pyruvate carboxylase assay

Pyruvate carboxylating activities in the absence and presence of acetyl-CoA were determined spectrophotometrically using a cou-pled reaction with malate dehydrogenase to detect oxaloacetate formation, as described previously [11]. Briefly, the assays were performed at 30 °C in 1 ml mixture containing 0.1 M Tris–HCl, pH 7.8, 20 mM NaHCO$_3$, 6 mM MgCl$_2$, 1 mM MgATP, 0.22 mM NADH, 10 mM sodium pyruvate and 5 units of MDH. The concen-trations of acetyl-CoA were varied from 0 to 150 μM. The data were analysed by non-linear regression fits to Eq. (1) [5]:

$$^{app}k_{cat} = k_{0cat} + k_{cat}/(1 + (K_a/[\text{acetyl CoA}])^n) \tag{1}$$

where $^{app}k_{cat}$ is the measured k_{cat} at each concentration of acetyl CoA, k_{cat} is the catalytic rate constant at saturating acetyl CoA, K_a is the activation constant for acetyl CoA and n is the Hill coefficient of the activation by acetyl CoA. The catalytic rate constant in the absence of acetyl CoA is k_{0cat} and in the case of the wild-type enzyme the value shown in Table 2 was determined experimentally as a mean and standard deviation of three measurements, since although the value estimated from the fit was similar to the experimental value it had a relatively large error. In the cases of the mutants, the values of k_{0cat} reported are the values estimated from non-linear regression fits of the $^{app}k_{cat}$ vs [acetyl CoA] data to Eq. (1), although the data analysed included an experimental value of k_{0cat}.
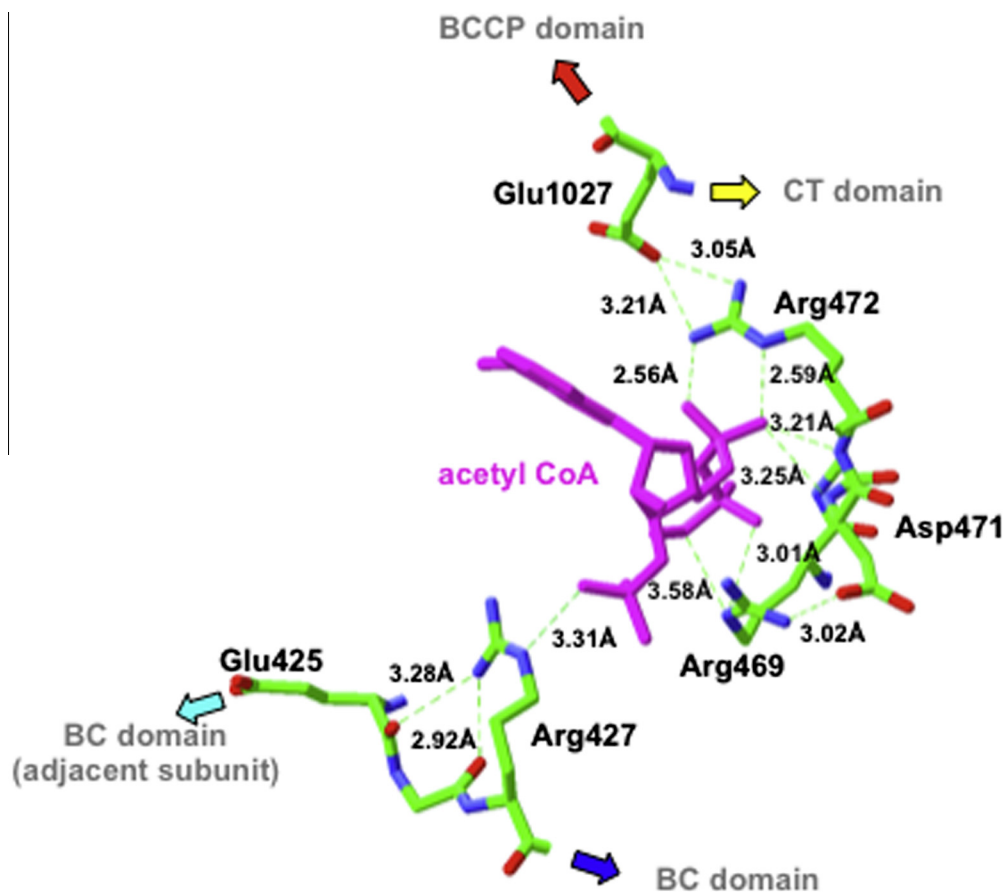
Fig. 2. Residues in the allosteric domain of RePC that directly interact with acetyl CoA and those that interact with these residues. Residues are taken from the structure of RePC determined by Lietzan et al. (2011) (PDB: 3TW6). Dashed lines indicate potential hydrogen bonds and the coloured arrows indicate connexions from the allosteric domain to other parts of the enzyme structure.

Table 1
Mutagenic oligonucleotide primers used to generate RePC mutants.

Mutant construct	Forward primer	Reverse primer
R469S	5′-gca-ggt-caa-gag-cca-gga-ccg-3′	5′-cgg-tcc-tgg-ctc-ttg-acc-tgc-3′
R469K	5′-cag-cag-gtc-aag-aaa-cag-gac-cgc-gcg-3′	5′-cgc-gcg-gtc-ctg-ttt-ctt-gac-ctg-ctg-3′
D471A	5′-aag-cgc-cag-gcg-cgc-gcg-acg-3′	5′-ctg-cgc-gcg-cgc-ctg-gcg-ctt-3′
D1018A	5′-acg-ggt-tgg-cgg-ccg-gcg-agg-agc-t-3′	5′-agc-tcc-tcg-ccg-gcc-gcc-aac-ccg-t-3′
E1027A	5′-tcg-ccg-aca-tcg-cga-agg-gca-aga-c-3′	5′-gtc-ttg-ccc-ttc-gcg-atg-tcg-gcg-a-3′
E1027R	5-tgt-tcg-ccg-aca-tca-gga-agg-gca-aga-cgc	5′-gcg-tct-tgc-cct-tcc-tga-tgt-cgg-cga-aca-3′

Table 2
Effects of mutations on acetyl CoA-activation of pyruvate carboxylation.

Wild-type (WT)/mutant form of RePC	k_{0cat} (−acetyl CoA) (s^{-1})	k_{cat} (+acetyl CoA) (s^{-1})	Hill coefficient	K_a (μM)
WT	0.18 ± 0.01	17.6 ± 0.1	2.7 ± 0.2	7.7 ± 0.6
R469S	1.61 ± 0.15	11.3 ± 0.3	2.3 ± 0.2	25.4 ± 0.8
R469K	0.68 ± 0.16	6.05 ± 0.44	0.9 ± 0.1	39.9 ± 6.0
D471A	0.11 ± 0.01	0.105 ± 0.002	N.D.	N.D.
E1027A	3.94 ± 0.28	14.9 ± 0.4	3.0 ± 0.3	12.9 ± 0.5
E1027R	1.18 ± 0.07	2.71 ± 0.18	2.3 ± 0.4	74.8 ± 6.4
D1018A	2.17 ± 0.25	12.3 ± 0.3	2.1 ± 0.2	10.9 ± 0.5

2.4. Bicarbonate-dependent MgATP cleavage assay

Bicarbonate-dependent MgATP cleavage activities in the presence and absence of acetyl-CoA at saturating MgATP were determined by coupled assay with pyruvate kinase and lactate dehydrogenase as described previously [11]. Briefly, the reactions were performed at 30 °C in 1 ml mixture containing 0.1 M Tris–HCl, pH 7.8, 20 mM NaHCO$_3$, 5 mM MgCl$_2$, 1 mM ATP, 0.22 mM NADH, 10 mM phosphoenolpyruvate, 5 units of pyruvate kinase and 4 units of lactate dehydrogenase. When acetyl CoA was present in the assay, a concentration of 0.25 mM was used.

3. Results

3.1. Arg469 mutations

To examine whether the guanidinium group of the side chain of Arg469 participates binding acetyl-CoA as shown in Fig. 2, this residue was mutated to serine (R469S) such that the interaction would be disrupted. To see if the length of the side chain of Arg469 affects participation in such an interaction, R469K mutant was also generated and characterised. Fig. 3 shows the activation by acetyl CoA of the pyruvate carboxylation reaction in the wild-type enzyme and R469S mutant. As shown in Table 2, the R469S mutant showed a 9-fold increase in the acetyl CoA-independent activity (k_{0cat}) and a 3.3-fold increase in K_a compared to the wild-type enzyme. There was only a small effect on the Hill coefficient and k_{cat} was reduced to about 70% of the value
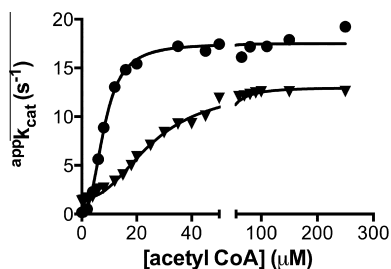
Fig. 3. Activation of pyruvate carboxylation by acetyl CoA in (●) wild-type RePC and (▼) R469S. Lines are non-linear regression fits of the data to Eq. (1).

for the wild-type enzyme. In contrast, the R469K mutation resulted in a 3.8-fold increase in k_{0cat} and a 5.2-fold increase in K_a compared to the wild type enzyme. The value of k_{cat} of R469K was 37% of that in wild-type RePC, but the largest difference between R469S and R469K mutants was that the cooperativity of acetyl CoA activation of R469K was abolished, with the value of the Hill coefficient being close to 1. The net result of the mutations was to reduce the effectiveness of activation of RePC-catalysed pyruvate carboxylation by acetyl CoA $((k_{cat}/K_a)/k_{0cat})$ to 0.28 (R469S) and 0.22 (R469K) compared to 12.70 in wild-type RePC.

As shown in Table 3, the effect of the R469S mutation on bicarbonate-dependent MgATP cleavage was to increase both k_{0cat} (34-fold) and k_{cat} (7.1-fold) relative to the values for wild-type RePC. Similar, but slightly smaller effects were seen in the R469K mutant, with k_{0cat} being 23-fold greater than the wild-type value and k_{cat} being 5.3-fold greater.

3.2. Asp471 mutation

The mutation of Asp471 to alanine resulted in a relatively small reduction in k_{0cat} of about 40%, but completely abolished the activation of the pyruvate carboxylation reaction by acetyl CoA (see Table 2).

3.3. Glu1027 mutations

As shown in Table 2, E1027A showed a 22-fold increase in k_{0cat} and a 1.7-fold increase in K_a compared to those of the wild-type enzyme. The E1027A mutant showed only a marginal effect on the Hill coefficient, and the k_{cat} was reduced slightly to about 85% of the value for the wild-type enzyme. Thus, the effect of the E1027A substitution was to reduce the effectiveness of activation by acetyl CoA to approximately 0.29 compared to 12.7 in the wild-type RePC. The E1027R mutant showed a lesser increase in k_{0cat} (6.6-fold) but a larger increase in K_a (10-fold) compared to wild-type RePC. There was again little effect on the Hill coefficient, but k_{cat} was reduced to only 16% of the value for the wild-type enzyme. Again, the net result in E1027R was to greatly decrease the effectiveness of activation of the enzyme-catalysed pyruvate carboxylation by acetyl CoA to 0.03.

As shown in Table 3, the effect of the E1027A mutation on bicarbonate-dependent MgATP cleavage was to dramatically increase both k_{0cat} (56-fold) and k_{cat} (7.7-fold) relative to those values for wild-type RePC. Similar, but considerably smaller effects were seen in the E1027R mutant, with k_{0cat} being 6.7-fold greater than the wild-type value and k_{cat} being 2.8-fold greater.

3.4. Asp1018 mutation

As shown in Table 2 the mutation D1018A resulted in a 12-fold increase in k_{0cat} and a 1.4-fold increase in K_a compared to the wild-type enzyme. The Hill coefficient and k_{cat} were reduced to 78% and 70% respectively of those values for the wild-type enzyme. The effectiveness of activation by acetyl CoA was 0.52.

As shown in Table 3, the effect of the D1018A mutation on bicarbonate-dependent MgATP cleavage was to dramatically increase both k_{0cat} (35-fold) and k_{cat} (8.3-fold) relative to the values for wild-type RePC.

4. Discussion

In summary there are mainly two types of result that are produced by mutation of the residues. Firstly, there are the mutations that affect acetyl CoA action by producing relatively large reductions in k_{cat} and/or increases in K_a in the pyruvate carboxylation reaction relative to wild-type enzyme (R469S/K, D471A, E1027R). These can be explained in terms of disruption of acetyl CoA binding and/or action, either by disruption of a direct interaction between the mutated residue and acetyl CoA or disruption of acetyl CoA interaction with other residues. The other type of result is where the mutations have resulted in increased pyruvate carboxylating activity in the absence of acetyl CoA and increased bicarbonate-dependent MgATP cleavage activity both in the presence and absence of acetyl CoA (R469S/K, E1027A/R, D1018A). As can be seen, some mutations produced both types of result.

To deal with mutations producing the first type of result, as can be seen in Figs. 2 and 4, the side chain guanidinium group of Arg469 and the α-amide of Asp471 interact directly with acetyl CoA. In addition, in the presence of acetyl CoA, both Glu1027 and Asp471 interact with the acetyl CoA-binding residues Arg472 and Arg469 respectively. It is easy to understand how the mutation R469S could result in a reduction of binding of acetyl CoA and hence produce the increase in K_a. However, the more conservative mutation R469K actually produced a greater increase in K_a than R469S, abolished the cooperativity of action acetyl CoA and reduced k_{cat} more than R469S. These effects of mutation of Arg469 do however, mirror the effects of mutation of the other major acetyl CoA-binding residues, Arg427 and Arg472 [5]. Mutation of Arg427 to serine or lysine produced increases in K_a for acetyl CoA of 15- and 76-fold relative to wild-type respectively and the R427K mutant also resulted in a loss of cooperativity of the action of acetyl CoA. Mutation of Arg472 to serine or lysine resulted in increases in K_a of 203- and 252-fold, respectively. Adina-Zada et al. [5] rationalised these observations by suggesting that the mutation of the arginine residues (Arg427 and Arg472) to serine removed any interaction between these residues and acetyl CoA, leaving acetyl CoA to be positioned in the binding pocket by the remaining interacting residues. Mutation of the arginine to lysine however suggested that whilst lysine could interact with acetyl CoA, lysine could not form the interactions with other residues in the allosteric binding site that arginine can, thus resulting in incorrect positioning of acetyl CoA in the binding site. This rationale may also apply to Arg469, which also interacts with Asp471 (see Fig. 4).

To some degree the very pronounced effect of the D471A mutation on acetyl CoA-activation of the RePC may be explained by the

Table 3
Effects of mutations on bicarbonate-dependent MgATP cleavage in the absence of pyruvate.

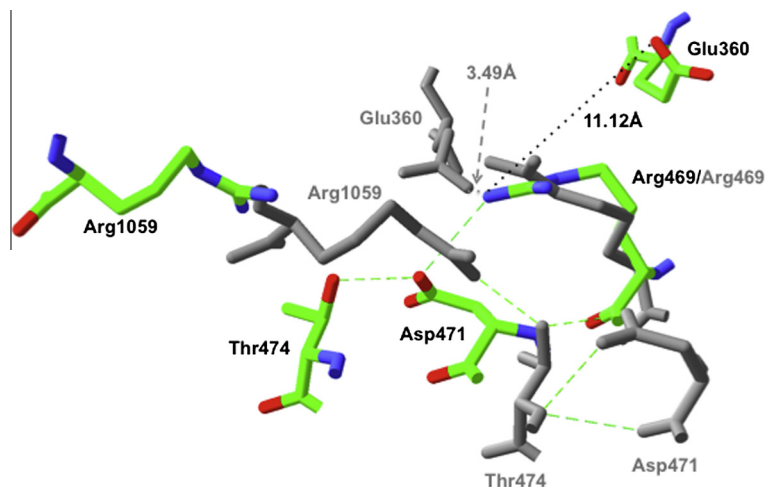| WT/mutant form of RePC	k_{0cat} (−acetyl CoA) (s^{-1})	k_{cat} (+acetyl CoA) (s^{-1})
WT	0.058 ± 0.002	0.53 ± 0.01
R469S	1.98 ± 0.15	3.75 ± 0.15
R469K	1.33 ± 0.16	2.82 ± 0.03
E1027A	3.25 ± 0.13	4.10 ± 0.03
E1027R	0.39 ± 0.04	1.49 ± 0.03
D1018A	2.050 ± 0.002	4.38 ± 0.09

Fig. 4. Relative positions of Glu360, Arg469, Asp471, Thr474 and Arg1059 residues in the RePC subunit with acetyl CoA bound (coloured residues/black labels) and without acetyl CoA bound (grey residues/grey labels) obtained by aligning Arg469 from both subunits in the structure of RePC obtained by Leitzan et al. (PDB: 3TW6) where the positions of all the residues are well defined (which is not the case in 2QF7). Dashed lines indicate potential hydrogen bonds and the distances indicated are between the guanidinium of Arg471 and the carboxyl of Glu360 in the presence and absence of acetyl CoA.

loss of interaction with Arg469. As can be seen in Fig. 4, Asp471 (including its α-amide) is capable of a large degree of displacement in the absence of acetyl CoA to a position where its carboxyl group no longer interacts with Arg469. The abolition of the interaction with Arg469 in D471A, may result in the displacement of D471A and loss of binding of its α-amide with acetyl CoA. The dramatic effects of mutation of Asp471 on acetyl CoA activation of RePC indicate that any reductions in acetyl CoA binding affinity are probably accompanied by loss in the ability of acetyl CoA to activate the enzyme, even at saturating acetyl CoA concentrations. This points to a very important role for Asp471 in the mechanism of activation by acetyl CoA, in addition, the reduction in k_{0cat} resulting from mutation of Asp471 suggests it also plays some role in basal catalysis, however Asp471 has not been reported to play a direct catalytic role in RePC. Thus the roles of Asp471 in catalysis and acetyl CoA-activation of RePC warrant further investigation.

As can be seen in Fig. 2, Glu1027 interacts with Arg472 in the subunit of RePC with acetyl CoA bound. The location of Glu1027 relative to Arg472 in subunits of RePC where acetyl CoA is not bound is unclear due to imprecise positioning (due to disorder) of Glu1027 and/or Arg472 in the structures of these subunits (in PDB files 2QF7, 3TW6 and 3TW7) [6,7]. The imprecise positioning of some residues in one structure but not another explains why Figs. 2 and 4 use PDB file 3TW6 and Fig. 5 uses 2QF7. Again, one result of mutation of Glu1027 to arginine may be due to mis-positioning of Arg472 in the binding of acetyl CoA, owing to charge-repulsion between the positive charge of arginine in the E1027R mutant and this residue (which would not occur with E1027A). This would then produce a reduction in the affinity of acetyl CoA binding and loss of activation of the enzyme as previously argued for lysine mutations of Arg427 and Arg472 [5].

Increased values of k_{0cat} produced by some mutations are not, by definition, caused by effects on acetyl CoA binding and thus, it is likely that these mutations have affected the interactions of these residues with others that occur in the absence of acetyl CoA. The obvious approach would seem to be to examine what interactions these residues make with others in the subunits of RePC without acetyl CoA bound compared to subunits with bound acetyl CoA. As seen in Fig. 4, Arg469 interacts with Glu360 in the absence of bound acetyl CoA and similarly Asp1018 interacts with Arg427 (Fig.5) (differential interactions between Glu1027 and
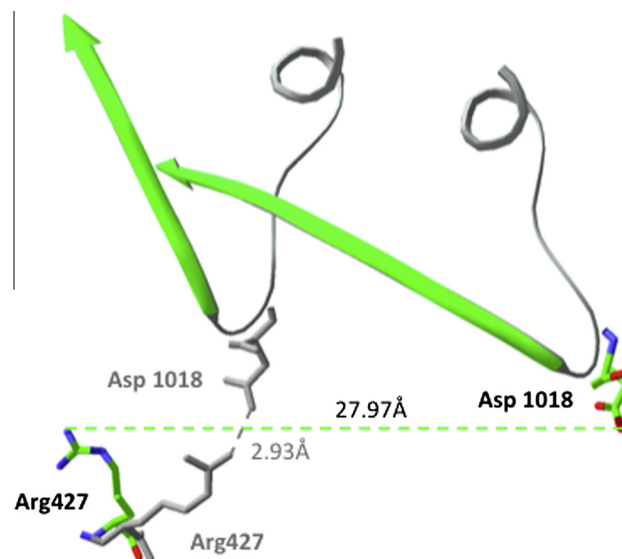

Fig. 5. Positioning of Arg427 and Glu1018 in the RePC subunit with acetyl CoA bound (coloured residues/black labels) and without acetyl CoA bound (grey residues/grey labels. Figure obtained by aligning the ATPγS bound in the subunit containing the acetyl CoA analogue, ethyl CoA, with that in the subunit with no ethyl CoA bound (from PDB file 2QF7; [7]).

Arg472 in RePC subunits with and without acetyl CoA bound cannot be determined because of the imprecise positioning of these residues described above). The implication being that acetyl CoA binding results in the loss of such interactions and releases any constraints that these interactions have on conformational changes required for catalysis in the absence of the activator. However the crystal structure of the RePC tetramer is very asymmetrical, even in the absence of acetyl CoA [6,7], giving rise to the possibility that it is this asymmetry that results in the differential positioning of the residues discussed above, not acetyl CoA binding. Comparison of the positions of these residues in the RePC tetramer structure determined with acetyl CoA bound with those in the structure of RePC tetramer without acetyl CoA bound is not possible because of the low resolution of the latter structure [6,7].

The crystal structures of other pyruvate carboxylase tetramers that have been determined have not exhibited this very marked asymmetry [8–10,16]. The crystal structure of SaPC has been determined in the absence of acetyl CoA (PDB: 3BG5) and also in its presence (PDB: 3H08), where the activator is bound to all four subunits. The residues in SaPC that correspond to Glu360, Arg427, Arg469, Arg472, Asp1018, Glu1027 and Arg1059 in RePC from sequence alignments are Leu353, Arg420, Ser462, Arg465, Asn1012, Asp1021 and Arg1053, respectively. The positions of pairs residues in SaPC subunits that correspond to those that interact in RePC subunits with no acetyl CoA bound were examined. None of the residues within these pairs are close enough to interact with each other in either of the SaPC structures and their positions relative to each other are not markedly different between the two structures. This is suggestive that in RePC it is not primarily acetyl CoA binding that changes the positions of these residues, rather it is the asymmetry of the tetramer. Unlike Arg1059 in RePC, Arg1053 in SaPC interacts directly with acetyl CoA and in the structure without acetyl CoA, it interacts directly with Asp465 that is equivalent to Asp471 in RePC.

So what is the relevance of these interactions between residues that occur in the asymmetrical tetramer of RePC? Although the crystal structure of the SaPC tetramer is more symmetrical than that of RePC, it still exhibits asymmetry [16]. In addition, Tong and co-workers have recently presented cryo-electron microscopy evidence that the SaPC tetramer undergoes transitions between symmetrical and asymmetrical conformations, depending on the stage of the enzyme-catalysed reaction, and that the half-of-the-sites activity alternates between pairs of subunits on opposite faces of the tetramer [17]. Thus, the difference between the crystal structure of the RePC tetramer and those of the SaPC and LmPC tetramers is likely due to enhanced stabilization of the asymmetrical conformer in RePC. Tong and coworkers [17] have speculated that differences in inter-tetramer contacts between SaPC and RePC in the crystal may result in stabilization of the different tetrameric conformers. It is possible that the interactions between residues in the allosteric domain of RePC that do not occur in SaPC may also contribute to this enhanced stabilization of the asymmetrical conformation of the RePC tetramer (Arg469–Glu360; Arg427–Asp1018).

In addition, these interactions between residues that are evident in the asymmetrical conformer may constrain the conversion to the symmetrical conformer. Thus mutation of these residues so as to remove such interactions would relieve these constraints, allowing freer inter-conversion between the two conformers. This inter-conversion may be much more rate-limiting in the absence of acetyl CoA than in its presence and so the effects of the mutations would be much more pronounced on the acetyl CoA-independent activity. It is interesting to note that the acetyl CoA-independent activity of SaPC is very much higher than that of RePC both in terms of the percentage of the activity in the presence of acetyl CoA (about 25%) and in absolute terms, 5 s^{-1} (at room temperature) [10].

The MgATP cleavage reactions were performed in the absence of pyruvate and the decarboxylation of the carboxybiotin, formed in the BC domains, in the CT domains to complete the catalytic cycle becomes rate-limiting [18,19], even in the presence of acetyl CoA. In SaPC this reaction has been shown to be associated with a conversion from the asymmetrical conformer to the symmetrical conformer [17]. Thus the mutation of some of the residues in the current work may again remove interactions that stabilize the asymmetrical conformer and so enhance the rate of conversion to the symmetrical form and hence the overall rate of catalysis. The smaller effects of the mutations on the rate constants of the pyruvate carboxylation reactions compared to those on bicarbonate-dependent MgATP cleavage, even in the presence of acetyl CoA, suggest that binding of pyruvate may enhance the inter-conversion of the asymmetrical conformer to symmetrical conformer, making it less rate-limiting.

If the analysis of the effects of the mutation of residues that enhance enzymic activity is correct, this would suggest that one mode of activation of PC by acetyl CoA is that the binding of acetyl CoA to all four subunits in the PC tetramer, in conjunction with pyruvate, enhances the ease of inter-conversion of the two conformers of the tetramer. What is the purpose of stabilizing the structure in the asymmetrical conformation? This would serve to place the enzyme in the resting state in a conformation ready for biotin carboxylation, but also by increasing the stabilization of this conformer, this would make the enzyme more highly regulated by acetyl CoA, so that there is a tighter coupling between PC activity, fatty acid oxidation and anaplerosis.

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