Further studies on the localization of the reactive lysyl residue of pyruvate carboxylase

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We have shown the increase in the acetyl-CoA-independent activity of sheep liver pyruvate carboxylase following trinitrophenylation of a specific lysine residue (designated Lys-A) to be the result of a large stimulation of the first partial reaction and a slight stimulation of the second partial reaction catalysed by this enzyme. Like acetyl-CoA, the activators adenosine 3',5'-bisphosphate and CoA did not stimulate the catalytic activity of the trinitrophenylated enzyme in either the overall reaction or the first partial reaction. Conversely, trinitrophenylation had no effect on activation of the overall reaction and the second partial reaction by acetyl-phosphopantetheine. Protection experiments demonstrated that the presence of both acetyl-CoA and adenosine 3',5'-bisphosphate decreased the rate of loss of activity during exposure of sheep liver pyruvate carboxylase to trinitrobenzenesulphonic acid (TNBS), whereas acetyl-phosphopantetheine did not. 5'-AMP and acetyl-dephospho-CoA did not protect the enzyme against loss of activity, whereas the presence of adenosine 2',5'-bisphosphate only slightly decreased the rate of modification. This suggests that Lys-A interacts with the adenosine nucleotide portion of the acetyl-CoA molecule, specifically the 3'-phosphate moiety. Acetyl-CoA and adenosine 3',5'-bisphosphate were shown to protect pyruvate carboxylase from *Saccharomyces cerevisiae* against inhibition by TNBS. A [¹⁴C]acetyl-CoA-binding assay demonstrated that modification of Lys-A inhibits the binding of acetyl-CoA to *S. cerevisiae* pyruvate carboxylase, indicating that Lys-A is at or near the acetyl-CoA-binding site.

INTRODUCTION

Pyruvate carboxylase (PC; EC 6.4.1.1) from many sources possesses a reactive lysine residue whose integrity is essential for full enzymic activity. Modification of the enzyme with amino-group-selective reagents generally results in preferential loss of the catalytic activity that is stimulated by the allosteric activator acetyl-CoA [1–3]. The loss of acetyl-CoA-dependent activity is due to the modification of one lysine per active site [1,2], and we have designated this residue Lys-A. Acetyl-CoA has been shown to protect the enzyme against this modification [1–3]. Exposure of sheep PC to trinitrobenzenesulphonic acid (TNBS), in addition to inhibiting the rate of acetyl-CoA-stimulated carboxylation of pyruvate and the acetyl-CoA-dependent isotope-exchange reactions, causes an enhancement of the acetyl-CoA-independent carboxylation of pyruvate [3]. The separation of the catalytic and modifier sites observed for PC after modification with TNBS is considered to be good evidence for the allosteric nature of the interaction between acetyl-CoA and the enzyme (cf. [2,3]). However, it is unclear whether Lys-A forms part of the acetyl-CoA-binding site or is protected by a conformational change induced by activator binding.

On the basis of protection data, previous kinetic studies on sheep PC suggested that the ε-amino group of Lys-A may be involved in the enzyme-activator interaction [1]. The observation that trinitrophenylated (TNP-) sheep kidney PC still catalysed the hydrolysis of acetyl-CoA led Ashman *et al.* [3] to conclude that the activator molecule can bind to the chemically modified enzyme. They suggested that the protective effect of acetyl-CoA is due to Lys-A being inaccessible in the acetyl-CoA-activated conformation. Comparison of the activation of PC and phosphoenolpyruvate carboxylase by acyl derivatives of CoA led to

the suggestion that, in PC, Lys-A forms part of the activator-binding site, such that the ε-amino group of Lys-A interacts with the 3'-phosphate of acetyl-CoA [4].

Equilibrium kinetic studies have shown that the activator acetyl-CoA influences events of both the first and second partial reactions catalysed by PC [5], described by eqns. (1) and (2) respectively:

and that these effects are due to the binding of one acetyl-CoA molecule per active site [6]. We have investigated the location of Lys-A by examining the overall reaction and the two partial reactions catalysed by TNP-(sheep liver PC) and the protective effect of various components of the acetyl-CoA molecule on TNBS modification of PC from sheep liver and *Saccharomyces cerevisiae*. The results are consistent with those of a direct acetyl-CoA-binding assay which indicated that Lys-A is at or near the acetyl-CoA-binding site of yeast PC.

MATERIALS AND METHODS

Enzyme preparation and assay procedures

Pyruvate carboxylase was purified from freeze-dried sheep liver mitochondria as previously described [7] to specific activities of 17–35 units/mg of protein, and from baker's yeast (*S. cerevisiae*) as described by Lim *et al.* [8]. One unit of enzyme activity is defined as the amount of enzyme required to catalyse the formation of 1 μmol of oxaloacetate/min.

Abbreviations used: PC, pyruvate carboxylase (EC 6.4.1.1); TNBS, trinitrobenzenesulphonic acid; TNP, trinitrophenylated.

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The assay systems and procedures used for measuring the activity of PC in the overall reaction and the ATP:P_i and pyruvate:oxaloacetate exchange reactions were as described previously [9], except that Tris buffer was replaced by *N*-ethylmorpholine (redistilled) and yeast PC was assayed at pH 8.0. Where indicated, acetyl-CoA was omitted from the assay system or replaced with another activator at the concentration stated. To minimize the effect of dilution inactivation during the overall reaction in the absence of any activator [5] and with acetyl-phosphopantetheine, the enzyme was present at a higher concentration and the assay time was decreased to 30 s. When the ATP:P_i exchange reaction was carried out with acetyl-CoA or CoA, 0.25–0.5 unit of PC was present per assay. In the absence of any activator and with adenosine 3',5'-bisphosphate, 1.25–2.5 units of PC were added.

Chemical modification of PC

Chemical modification with TNBS was carried out for sheep PC in 0.1 M-*N*-ethylmorpholine/HCl, pH 8.4, for up to 15 min and for yeast PC in 0.1 M-Tris/HCl, pH 7.2, for up to 80 min at 30 °C. The buffer systems and pH were chosen for reasons of enzyme stability and did not affect the modification reaction. Modification was initiated by the addition of TNBS to 0.1 mM and 0.5 mM for sheep and yeast enzyme respectively and quenched by removal of samples into L-lysine [3].

Acetyl-CoA-binding assay

Acetyl-CoA binding was determined by measuring the ability of PC to retard the progress of [¹⁴C]acetyl-CoA through an *M*_w 30000-cut-off ultrafiltration membrane [10,11]. Yeast PC at concentrations of 0.5, 1.0 and 2.0 mg/ml was combined with [¹⁴C]acetyl-CoA (sp. radioactivity 10⁷ c.p.m./μmol) at a final concentration of 70 μM in 0.1 M-Tris/HCl, pH 7.2, in Amicon MPS-1 ultrafiltration tubes and immediately centrifuged at 1500 *g* for 10 min at 4 °C. The radioactivity in samples of the filtrate was determined and compared with control samples where PC was omitted. In some cases yeast PC was incubated with TNBS until 40 % of the initial enzymic activity remained, and the remaining TNBS was quenched with L-lysine. Samples which had not been exposed to TNBS were pretreated with TNP-lysine to minimize the effect of any non-specific adsorption to the ultrafiltration tubes.

Preparation of acetyl-CoA and fragments of the acetyl-CoA molecule

Acetyl-CoA was prepared as previously described [12] and further purified for the binding assays on an Anion Spheri-10 column (Brownlee Labs.) pre-equilibrated in 5 mM-triethylamine acetate/10 % (v/v) acetonitrile, followed by elution with a linear gradient to 450 mM-triethylamine acetate/10 % (v/v) acetonitrile. [¹⁴C]Acetyl-CoA was prepared by using [¹⁴C]acetic anhydride (Amersham). [¹⁴C]Acetic anhydride (9 nmol) was added to aqueous reduced CoA (36 nmol) at pH 7.2. After 20 min at room temperature, non-radioactive acetic anhydride (40 nmol) was added and allowed to react for a further 20 min to drive the reaction to completion. Purified acetyl-CoA (5 μmol) was added as carrier, and the mixture was purified by anion exchange as described above.

Phosphopantetheine was prepared from D-pantetheine (Sigma Chemical Co.) by the procedure of Baddiley & Thain [13], and subsequently acetylated by the method described for acetylation of CoA [12]. Acetyl-dephospho-CoA was prepared from acetyl-CoA by using nuclease P1 (3'-ribonucleotide phosphohydrolase from *Penicillium citrinum*; Sigma Chemical Co.). Acetyl-CoA (2.5 mM, in 50 mM-Tris/HCl, pH 7.4) was incubated at room temperature with 1.5 units of nuclease P1/ml and the release of

P_i was monitored by the method of Lin & Morales [14]. After 4 h the reaction was complete, and the reactants were separated on DEAE-cellulose with a linear gradient of 0.08–0.3 M-LiCl in 3 mM-HCl [15]. Acetyl-dephospho-CoA was eluted with 0.1 M-LiCl (as distinct from acetyl-CoA, which was eluted with 0.16 M-LiCl) and was desalted by Sephadex G-10 chromatography. In two different chromatographic systems [12], the material gave a single spot which was distinct from acetyl-CoA and had an *R_f* value identical with that of an acetylated dephospho-CoA sample obtained commercially.

Other materials were as previously described [5,9] or of analytical reagent grade.

RESULTS

Influence of fragments of the acetyl-CoA molecule on various activities of sheep liver PC

Compounds chosen to represent various fragments of the acetyl-CoA molecule were used as activators of sheep liver PC in the overall reaction and the first and second partial reactions, as summarized in Table 1. The activator concentrations shown are those which gave maximal activity and hence were those used in subsequent work. The adenosine nucleotide portion of the acetyl-CoA molecule stimulates the activity of the first partial reaction (eqn. 1), while having no effect on the second partial reaction (eqn. 2), whereas the reverse is the case for the acetyl-phosphopantetheine group.

Influence of fragments of the acetyl-CoA molecule on various activities of trinitrophenylated sheep liver PC

The effect of TNBS modification of sheep liver PC on the ability of the acetyl-CoA fragments to activate the overall reaction catalysed by the enzyme is shown in Fig. 1. Acetyl-CoA-stimulated activity is markedly decreased by trinitrophenylation, whereas the acetyl-CoA-independent activity is enhanced, as is seen with sheep kidney PC [3]. The catalytic activity of the native enzyme in the absence of acetyl-CoA under the conditions used here is approx. 2 % of the acetyl-CoA-stimulated rate, and thus the increase in activity after modification, although relatively large, is not significant in the samples assayed in the presence of acetyl-CoA. The ability of adenosine 3',5'-bisphosphate and CoA to stimulate TNP-(sheep liver PC) is lost similarly, whereas

Table 1. Ability of fragments of the acetyl-CoA molecule to activate the overall reaction and the two partial reactions catalysed by sheep liver PC

The activity of sheep liver PC was measured as described in the Materials and methods section in the overall reaction and in the ATP:P_i and pyruvate:oxaloacetate exchange reactions with acetyl-CoA replaced by the compound indicated. The results are expressed as a percentage of the rate observed in the presence of saturating concentrations of acetyl-CoA: * not determined.

Activator	Concn. (mM)	Overall reaction	ATP:P _i exchange (Eqn. 1)	Pyruvate:oxaloacetate exchange (Eqn. 2)
CoA	10.0	74.5	88.7	0.0
Adenosine 3',5'-bisphosphate	5.0	8.8	16.7	*
Acetyl-phosphopantetheine	5.0	3.9	0.0	55.8

Fig. 1. Effect of acetyl-CoA and components of the acetyl-CoA molecule on TNBS-modified sheep liver PC

Enzyme was incubated with TNBS and samples taken at various time intervals were assayed as described in the Materials and methods section for activity in the absence of any effector molecule ($\square$) and in the presence of 0.25 mM-acetyl-CoA ($\triangle$), 10 mM-CoA ($\blacktriangle$), 5 mM-adenosine 3',5'-bisphosphate ($\bullet$) or 5 mM-acetyl-phosphopantetheine ($\blacksquare$); ($\circ$) no TNBS.

Fig. 2. Effect of acetyl-CoA and components of the acetyl-CoA molecule on the ATP: P_i exchange activity of TNBS-modified sheep liver PC

Enzyme was incubated with TNBS, and samples taken at various time intervals were assayed as described in the Materials and methods section for ATP: P_i exchange activity in the absence of any activator ($\square$) and in the presence of 0.25 mM-acetyl-CoA ($\triangle$), 10 mM-CoA ($\blacktriangle$) or 5 mM-adenosine 3',5'-bisphosphate ($\bullet$).

Fig. 3. Effect of acetyl-CoA and acetyl-phosphopantetheine on the pyruvate:oxaloacetate exchange activity of TNBS-modified sheep liver PC

Enzyme was incubated with TNBS, and samples taken at various time intervals were assayed as described in the Materials and methods section for pyruvate:oxaloacetate exchange activity in the absence of any activator ($\square$) and in the presence of 0.25 mM-acetyl-CoA ($\triangle$) or 5 mM-acetyl-phosphopantetheine ($\blacksquare$).

Table 2. Effect of acetyl-CoA and fragments of the acetyl-CoA molecule on the rate of inactivation of sheep liver and yeast PC by TNBS

Sheep liver or yeast PC was incubated with TNBS as described in the Materials and methods section, except that the indicated additions were allowed to equilibrate with the enzyme for 10 min at 30 °C before modification was initiated by the addition of TNBS. Samples taken at various time intervals were assayed for residual enzymic activity in the presence of acetyl-CoA as described in the Materials and methods section. The rate of inactivation, k' , was calculated from a plot of $\log(\% \text{ residual activity})$ versus modification time [1].

Added component	Concn. (mM)	$10^2 k'$ (min^{-1})
Sheep liver PC		
None	—	7.41
Acetyl-CoA	0.5	0.214
Adenosine 3',5'-bisphosphate	5.0	2.64
Adenosine 2',5'-bisphosphate	10.0	1.74
5'-AMP	5.0	5.03
Acetyl-dephospho-CoA	5.0	8.73
Acetyl-phosphopantetheine	10.0	8.12
Acetyl-phosphopantetheine	5.0	6.76
Yeast PC		
None	—	0.550
Acetyl-CoA	0.5	0.138
Adenosine 3',5'-bisphosphate	0.5	0.566
	5.0	0.330

activation by acetyl-phosphopantetheine is not altered (Fig. 1). Fig. 2 shows the effect of the components of the acetyl-CoA molecule which activate the first partial reaction [eqn. (1); cf. Table 1] on ATP: P_i exchange activity of TNP-(sheep liver PC). Stimulation of the reaction by acetyl-CoA, adenosine 3',5'-bisphosphate and CoA is decreased in a way which parallels the effect on the overall activity. The slow exchange of ^{32}P between ATP and P_i which is detected in the absence of acetyl-CoA is enhanced by TNBS modification to the same extent as the corresponding overall activity (cf. Fig. 1).

Activation of the second partial reaction by acetyl-CoA, i.e. the pyruvate:oxaloacetate exchange activity [eqn. (2)], is decreased after modification of sheep liver PC with TNBS, whereas the acetyl-CoA-independent exchange activity is slightly increased (Fig. 3), as with sheep kidney PC [3], although not to the extent seen in the overall reaction. It is also apparent that, although acetyl-CoA stimulation is decreased, the degree of activation by acetyl-phosphopantetheine remains constant, regardless of the extent of modification. This effect is also observed in the overall reaction (Fig. 1) and indicates that the binding of the compound to the enzyme has not been altered by trinitrophenylation of Lys-A. These results suggest that the loss of acetyl-CoA-stimulated overall activity after modification of Lys-A is primarily a consequence of the adenosine nucleotide portion of acetyl-CoA being unable to activate the first partial reaction.

Protection of sheep liver PC against TNBS modification by fragments of the acetyl-CoA molecule

The ability of fragments of the acetyl-CoA molecule to protect sheep liver PC against loss of catalytic activity during exposure to TNBS was examined as a function of ligand concentration, and the results are summarized in Table 2. As expected by analogy with pyruvate carboxylases from other sources [1–3,16], saturating concentrations of acetyl-CoA protect against loss of

Fig. 4. Effect of TNBS concentration on the loss of enzymic activity of yeast PC

(a) Yeast PC was treated with (○) 0, (●) 0.1, (□) 0.2, (■) 0.5, (△) 1.0 and (▲) 5.0 mM-TNBS, and samples taken during the time course were assayed for residual activity as described in the Materials and methods section. (b) Replot of the data in (a) in the form of $\log k'$ versus $\log[\text{TNBS}]$ for concentrations of 0.1–1.0 mM such that the slope of the line (0.7) gives the order of the reaction [1]. The rate of inactivation, k' , was determined as described in the legend to Table 2.

activity. Adenosine 3',5'-bisphosphate also decreases the rate of modification, whereas acetyl-phosphopantetheine does not. These results are consistent with the effects of these compounds on the overall and exchange activities of TNP-(sheep liver PC) (Figs. 1–3). It was not possible to examine protection afforded by CoA, since TNBS reacts with the thiol group of CoA at the concentration required (see also [16]). To characterize further the protection afforded by adenosine 3',5'-bisphosphate, we investigated the protective effects of analogues lacking the 3'-phosphate group. Table 2 shows that 5'-AMP and acetyl-dephospho-CoA did not significantly protect Lys-A against trinitrophenylation, whereas adenosine 2',5'-bisphosphate slightly decreased the rate of inactivation. The decrease in k' with adenosine 2',5'-bisphosphate is considerably less than that with the same concentration of adenosine 3',5'-bisphosphate, and may be due to a steric effect. This pattern of protection suggests that it is the 3'-phosphate group which is responsible for the protective effects of acetyl-CoA and adenosine 3',5'-bisphosphate in this case.

Although the results presented above are most simply explained if Lys-A is in or near the acetyl-CoA-binding site, on the basis of kinetic experiments one cannot completely eliminate the possibility that modification of Lys-A prevents the mediation of the effect of acetyl-CoA once bound. A direct determination of the ability of the modified enzyme to bind acetyl-CoA is the way to resolve the alternatives. The measurement of acetyl-CoA binding is complicated by a deacylase activity apparently associated with the enzyme [3,17]. Frey & Utter [18] measured this activity in chicken PC, and found that at 23 °C 4 nmol of acetyl-CoA was broken down/min per mg of protein. Although this activity is low compared with the carboxylation of pyruvate (15 $\mu\text{mol}/\text{min}$ per mg of protein), it is sufficient to interfere with the ability to determine accurately the binding constant of acetyl-

CoA in experiments taking longer than 2.5 min to perform at room temperature. To minimize the influence of any deacylase activity, a technique to detect acetyl-CoA binding must ideally be rapid, sensitive enough to allow low concentrations of PC, and must work at low temperatures. The robust PC from yeast is not affected by low temperatures [19], is structurally the best characterized of the PCs [8], and was therefore the enzyme of choice for this analysis.

Characterization of the effect of TNBS modification on yeast PC

Treatment of yeast PC with 1.0 mM-TNBS is known to result in loss of both the acetyl-CoA-dependent and -independent activities [2]. Further investigation of this system indicated that the inactivation reaction is pseudo-first-order with respect to time until 40 % of the initial activity remains (Fig. 4a). However, the overall rate of inactivation is lower than for the vertebrate enzymes [1,2], requiring considerably greater reaction times to achieve significant inhibition (80 min, compared with 5–10 min for sheep PC). The inactivation reaction is also pseudo-first-order with respect to TNBS concentration up to 1.0 mM, as is the case with sheep PC [1]. At higher concentrations of TNBS a divergence from linearity occurs, which is most likely to be due to modification at additional sites on the enzyme [1]. Replotting the data showing $\log k'$ as a function of $\log[\text{TNBS}]$ produces a straight line of slope 0.7 (Fig. 4b), suggesting that, like PC from vertebrate sources, inactivation of yeast PC is a consequence of modification of one catalytically essential lysine residue, Lys-A, per active site [1]. Subsequent studies were performed under conditions derived from these experiments, i.e. 0.5 mM-TNBS for 80 min to minimize modification of residues other than Lys-A. The ability of acetyl-CoA and adenosine 3',5'-bisphosphate to protect yeast PC against loss of catalytic activity during exposure to TNBS is shown in Table 2. The decrease in k' observed under the conditions used is similar to that seen with sheep liver PC (Table 2), suggesting that Lys-A has the same role in acetyl-CoA binding in PC from both sources.

Comparison of acetyl-CoA binding to yeast PC and to the TNP-enzyme

The ability of yeast PC to retard the progress of [¹⁴C]acetyl-CoA through an ultrafiltration membrane was used as an indicator of acetyl-CoA binding [10,11] and was applied to the question of whether TNBS-modified PC can bind acetyl-CoA. As described in the Materials and methods section, enzyme samples were mixed with a fixed concentration of acetyl-CoA and centrifuged at low temperature through an M_r -30000-cut-off membrane. The proportion of [¹⁴C]acetyl-CoA in the filtrate was then compared with control samples where no enzyme had been added. Fig. 5 shows a significant difference in acetyl-CoA binding ability when TNP-(yeast PC) was compared with untreated enzyme in this assay system. Control samples were able to retard radioactive acetyl-CoA more effectively than the same concentrations of TNP-PC, where at lower protein concentrations no binding was detected. Some retardation of [¹⁴C]acetyl-CoA occurred in the 2 mg/ml PC sample treated with TNBS, although this was less than the equivalent control and represents either the effect of remaining native enzyme in the sample or acetyl-CoA binding to modified enzyme with considerably decreased affinity. Some interference by a deacylase activity is evident, particularly at higher concentrations of PC, in that the untreated enzyme sample at 0.5 mg/ml had 51 % of binding sites occupied, whereas at 1.0 mg/ml and 2.0 mg/ml only 43 % and 22 % of binding sites were occupied respectively. It should be noted that the concentration of acetyl-CoA (70 μM) used in these binding assays was not intended to be saturating. We were interested in any

Fig. 5. Effect of TNBS modification on the capacity of yeast PC to bind acetyl-CoA

Yeast PC was treated with 0.5 mM-TNBS for 80 min as described in the Materials and methods section until 40 % of the initial enzymic activity remained. Samples of TNP-(yeast PC) (●) and native enzyme (□) at the concentrations shown were assayed for acetyl-CoA-binding ability as described in the Materials and methods section. The amount of [¹⁴C]acetyl-CoA in the filtrate is expressed as a percentage of that observed when enzyme was omitted from the binding solution. The data points represent averages of three determinations.

difference in acetyl-CoA-binding ability between modified and native enzyme, rather than an absolute value, and therefore chose a concentration which allowed detectable binding while minimizing the deacylase activity.

DISCUSSION

The observation that TNBS modification of yeast PC inhibits acetyl-CoA binding allows, for the first time, delineation between the two proposed mechanisms of TNBS-mediated inactivation of PC. We suggest that Lys-A forms part of the acetyl-CoA-binding site rather than being protected by a conformational change following activator binding. This conclusion is supported by the kinetic studies on the TNBS-modified sheep liver enzyme presented here. From the observation that the stimulation of the acetyl-CoA-independent activity is largely an effect on the first partial reaction (eqn. 1), it is reasonable to infer that Lys-A is in close proximity to the binding site for the adenosine nucleotide portion of the acetyl-CoA molecule. This is supported by the protection data for both yeast and sheep PC, which indicate that Lys-A is protected by this part of the activator molecule. In this context it is noteworthy that a crystallographic study has shown that the CoA-binding enzyme citrate synthase utilizes interactions between an arginine residue and the 3'-phosphate of CoA [20].

It appears that Lys-A is located in the vicinity of the acetyl-CoA-binding site such that its chemical modification does not prevent the interaction of the acetyl-phosphopantetheinyl moiety with the enzyme. This presents an explanation for the observation that acetyl-CoA is deacylated by the modified enzyme [3]. The kinetically determined dissociation constant of acetyl-CoA for the deacylation reaction is significantly higher than that for stimulation of catalytic activity [17]; thus a decreased affinity for acetyl-CoA following alteration of the binding site for the adenosine nucleotide part of the molecule caused by trinitrophenylation of Lys-A may result in loss of catalytic activity without altering deacylation. This is not inconsistent with the results of the acetyl-CoA-binding assay for yeast PC, since it is possible that the slight retention of [¹⁴C]acetyl-CoA by the highest concentration of TNP-(yeast PC) (Fig. 5) indicates acetyl-CoA binding with decreased affinity. Although this effect may be due to remaining native enzyme in the sample or to the net effect

of both phenomena, resolution of these possibilities is difficult. Analysis of altered kinetic parameters in the modified enzyme is complicated in a system containing both modified and native forms of a multimeric enzyme [21].

Trinitrophenylation of PC from different sources has been shown to have different effects on the properties of the catalytic sites [2,3,16]. However, the presence of one susceptible lysine residue per active site protected by acetyl-CoA is a constant feature for all these enzymes. Our conclusions as to the location of the reactive lysine residue in sheep liver and yeast PC are not consistent with those of Scrutton *et al.* [16] for chicken and rat liver PC; they concluded, on the basis of kinetic evidence alone, that the residue modified by TNBS is located some distance from the activator site. However, those workers point out that their results do not allow a definitive distinction between this and the possibility that the residue may be sufficiently close to the acetyl-CoA-binding site that the bulky trinitrophenyl group interferes with binding at the activator site. The observation that TNP-(yeast PC) at concentrations up to 1 mg/ml does not bind acetyl-CoA suggests, by analogy, that the latter explanation may in fact be correct. Unfortunately, the susceptibility to low temperatures, which is a feature of the chicken enzyme [22], makes a direct acetyl-CoA binding assay, which may resolve this question, difficult. Indeed, Frey & Utter [18] showed that the monomeric form of chicken liver PC observed at 0 °C failed to bind acetyl-CoA.

Although it appears that Lys-A is conserved between species, the increased time required for TNBS modification of yeast PC suggests that the chemical environment surrounding the residue is slightly different from that in the vertebrate enzyme; hence, in yeast Lys-A is less nucleophilic. Yeast PC is also different in that trinitrophenylation results in concurrent loss of the acetyl-CoA-independent activity [2]. The stimulation of this activity in TNP-(sheep PC), which has been localized to the first partial reaction, suggests that the trinitrophenyl group mimics an effect of acetyl-CoA in this environment.

The most satisfactory way to demonstrate the involvement of Lys-A in binding acetyl-CoA is by site-directed mutagenesis, once the residue has been identified in the protein sequence. PC from yeast is the obvious choice for such an analysis, as the complete amino acid sequence is known [8] and the necessary manipulations are relatively straightforward in this system. Further progress in this direction must await the isolation and identification of Lys-A, a complex task in a protein of this size.

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