The existence of multiple tetrameric conformers of chicken liver pyruvate carboxylase and their roles in dilution inactivation

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The time-dependent loss of enzymic activity and tetrameric structure of chicken liver pyruvate carboxylase (EC 6.4.1.1) after dilution below 2 units/ml was apparently monophasic and first-order. When examined over a range of initial enzyme concentrations, both activity and tetrameric structure decayed to equilibrium levels which were dependent on the initial concentration. The observed rate constants for the loss of enzymic activity (i) showed no apparent dependence on the initial enzyme concentration, and (ii) were of similar magnitude to the corresponding rate constants of dissociation. Computer simulations of the most likely kinetic model suggest that the predominant form of the dissociated enzyme is the monomer. Dilution of pyruvate carboxylase in the presence of the allosteric activator acetyl-CoA largely prevented the subsequent dissociation of the tetrameric molecule. In addition, acetyl-CoA was able to cause a degree of activation and reassociation when added after dilution inactivation had been allowed to occur. Electron-microscopic observation showed the treatment with avidin before dilution markedly decreased the degree of dissociation of the enzyme tetramer. This structure-stabilizing effect of avidin was dependent on preincubation of the concentrated enzyme solution with acetyl-CoA. We propose that, over a range of protein concentrations, the tetrameric enzyme exists in two forms that are in equilibrium, and that acetyl-CoA alters the equilibrium to favour the more compact form.

INTRODUCTION

When diluted below about 4 units/ml, pyruvate carboxylase (PC; EC 6.4.1.1) isolated from sheep tissues undergoes dilution inactivation, a time-dependent loss of enzyme activity, which is prevented in the presence of the allosteric activator acetyl-CoA (Ashman et al., 1972). Monitoring changes in quaternary structure by electron microscopy and rapid gel-filtration chromatography, Khew-Goodall et al. (1991) demonstrated that dissociation of the tetrameric form of sheep PC into dimers and monomers occurred during dilution inactivation. Whereas addition of acetyl-CoA halted both the loss of enzymic activity and the dissociation process, the activator caused a degree of reassociation, but was not able to restore activity (Ashman et al., 1972; Khew-Goodall et al., 1991). Chicken liver PC is known to undergo a similar process of cold inactivation during which the active tetramer dissociates into monomeric subunits (Irias et al., 1969). Cold inactivation is dependent on enzyme concentration (Scrutton and Utter, 1965), and can be prevented in the presence of sucrose (Utter et al., 1964) and acetyl-CoA (Irias et al., 1969).

We have employed rapid high-performance gel filtration together with conventional assay methods to determine the kinetics of (i) the loss of enzyme activity, (ii) the dissociation of the enzyme during the dilution inactivation process and (iii) the effect of acetyl-CoA on the activity and association state of chicken liver PC after dilution. Johannssen et al. (1983) found that at low ratios of avidin:PC (< 1:1), and in the presence of acetyl-CoA, one avidin molecule could bind both biotin molecules from a pair of adjacent subunits within a tetramer. We have used electron microscopy of chicken liver PC cross-linked with avidin to examine the effects of acetyl-CoA on quaternary structure during dilution inactivation.

MATERIALS AND METHODS

H.p.l.c.

The chromatography system, molecular-mass calibration and data analyses were as described by Khew-Goodall et al. (1991). The expected elution time for each form of the PC molecule (tetramer 8.75 min, trimer 9.45 min, dimer 10.25 min and monomer 11.75 min) was calculated from the calibration curve, based on a monomer molecular mass of 123 kDa, which was determined from the mobility relative to yeast PC (130 kDa; Lim et al., 1988) on SDS/PAGE.

Enzyme preparation and assay procedures

Chicken liver PC was purified as described by Goss et al. (1979) to a specific activity of 40 units/mg of protein, where 1 unit of enzyme catalyses the formation of 1 μmol of oxaloacetate/min at 30 °C. Enzymic activity was determined by using the spectrophotometric assay system described by Duggleby et al. (1982). The enzyme was stored in 0.1 M *N*-ethylmorpholine acetate, pH 7.0, containing 50 mM $(NH_4)_2SO_4$ and 1.6 M sucrose. Before use, PC was further purified by h.p.l.c. on a TSK G-3000 SW column in order to remove the enzyme from the storage buffer and have essentially tetrameric starting material. The PC fraction with the highest specific activity (160–170 units/ml) showed very minor contamination by other proteins (< 2%) on SDS/PAGE (Laemmli, 1970) and was used in all subsequent experiments.

The biotin content of PC solutions used in the experiments involving avidin was determined as described by Rylatt et al. (1977). The concentration of biotin-binding sites in the avidin solutions was determined by titration with [^{14}C]biotin using an analogous technique.

Abbreviation used: PC, pyruvate carboxylase (EC 6.4.1.1).

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Changes in association state and activity after dilution

PC was diluted to 2, 1 or 0.5 units/ml with 0.1 M Tris/HCl, pH 7.2, containing 0.1 M KCl, and at various times after dilution samples were injected on to the TSK G-3000 SW column to examine the association state, or assayed for remaining activity. Time courses were performed at 25 °C. When looking at the effect of acetyl-CoA on association state and activity, PC was diluted to 1 unit/ml in the above buffer, and at the times indicated in the Figure legends acetyl-CoA was added to a final concentration of 0.1 mM. The association state and enzymic activity at various times after the addition of acetyl-CoA were determined by h.p.l.c. or by assaying for remaining activity respectively. In these experiments, the h.p.l.c. running buffer also contained acetyl-CoA and the eluted protein was detected at 220 nm.

Analysis of kinetic data

Curve fitting to experimental data was performed with the non-linear least-squares regression program written by Duggleby (1981). The standard errors quoted for the parameters derived from these computer-fits were calculated as described in the program. Computer simulations of kinetic models were performed using KSIM, and apparent first-order rate constants were calculated from the simulated data using KFIT, a non-linear least-squares regression program. Both KSIM and KFIT were written by N. C. Millar, currently at the Department of Physiology, University of California, Los Angeles, CA, U.S.A. In KSIM the rate equations are solved by numerical integration using the Gear (1971) algorithm.

Electron microscopy

Tetrameric chicken liver PC was selected after h.p.l.c. gel filtration (65 units/ml, 46.7 μM biotin). Samples of enzyme were diluted with 0.1 M Tris/HCl, pH 7.2, containing 0.25 mM acetyl-CoA, or buffer alone. After equilibration for 20 min at room temperature ($\sim$ 25 °C), the required volume of avidin solution ($\sim$ 4 mg/ml, 228 μM biotin-binding sites) was added to each sample of PC so that the final ratios of biotin-binding sites: biotin were either 10:1 or 1:2, and the final concentration of PC was 16 units/ml. Control samples without avidin were prepared in parallel throughout. After incubation for a further 1 h, the samples were diluted to give, with ratios of avidin:PC of 10:1 and 1:2 respectively, solutions containing $\sim$ 10 μg/ml protein (0.12 unit/ml) and $\sim$ 40 μg/ml protein (0.5 unit/ml), concentrations which have been shown to be appropriate for electron microscopy under these conditions (Johannssen et al., 1983). The solutions were left at room temperature for 30–45 min, after which samples were prepared for electron microscopy.

Electron microscopy was carried out as described previously (Johannssen et al., 1983). Intact tetramers were readily identified by their shape and dimensions. The value of 100% was assigned to the number of tetramers ($\sim$ 350) observed in 1500 cm^2 prints where the enzyme sample was prepared in the presence of acetyl-CoA throughout. The number of tetramers in the same area of the micrographs for other treatments was expressed as a percentage of this control. The micrographs revealed the presence of a small amount of glutamate dehydrogenase, which, being readily distinguishable from PC by appearance and size (Mayer et al., 1980), did not affect the interpretation of the results.

Other materials were high-purity preparations as described by Duggleby et al. (1982).

RESULTS

Effect of dilution on PC activity

After dilution of chicken liver PC, the activity decayed with time to a final equilibrium level which was dependent on the initial activity (Figure 1). The loss of activity was apparently first-order, and the intercepts of the fitted curves on the ordinate were close to 100%, indicating that the reaction was monophasic. The parameters derived from the computer fits are given in Table 1. The observed first-order rate constants were similar regardless of the initial activity, indicating that the rate-limiting step for the inactivation process was not sensitive to enzyme concentration. The loss of activity is similar to that observed for sheep kidney PC (Ashman et al., 1972), but differs from sheep liver PC, where the activity decays to zero in a biphasic process (Khew-Goodall et al., 1991).

Effect of dilution on the association state of PC

Analysis of the association state of PC by gel filtration at various times after dilution demonstrated the presence of two major protein peaks (Figure 2). The first peak was eluted between 8.26 and 8.45 min and corresponded most closely to the expected elution time of the PC tetramer, calculated to be 8.75 min. The enzymic activity co-eluted with the first peak (Figure 2e). The

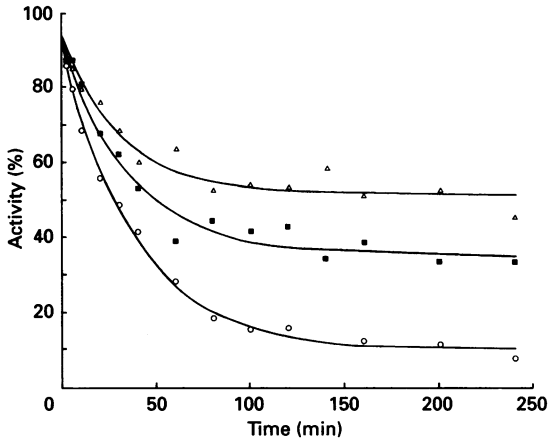


Figure 1 Time-dependent decay of PC activity on dilution

After dilution of PC in 0.1 M Tris/HCl, pH 7.2, containing 0.1 M KCl, to 2 (△), 1 (■) or 0.5 (○) units/ml, samples were removed and assayed for remaining activity as described in the Materials and methods section. The activity at each time was expressed as a percentage of the initial activity. The lines shown represent the non-linear regression fit of the data to a first-order exponential decay to a finite level of activity, calculated by using the non-linear least-squares regression program written by Duggleby (1981).

Table 1 Parameters derived from fitting curves that describe first-order decay to a finite level of activity to data describing the time-dependent loss of activity on dilution

Initial enzyme concn. (units/ml)	Intercept at zero time (% of initial activity)	Final equilibrium level of activity (% of initial activity)	Pseudo-first-order rate constant (min^{-1})
2	94.1 ± 4.9	51.9 ± 1.9	0.033 ± 0.006
1	91.5 ± 4.2	35.5 ± 1.7	0.027 ± 0.003
0.5	92.4 ± 3.8	10.5 ± 1.6	0.027 ± 0.002

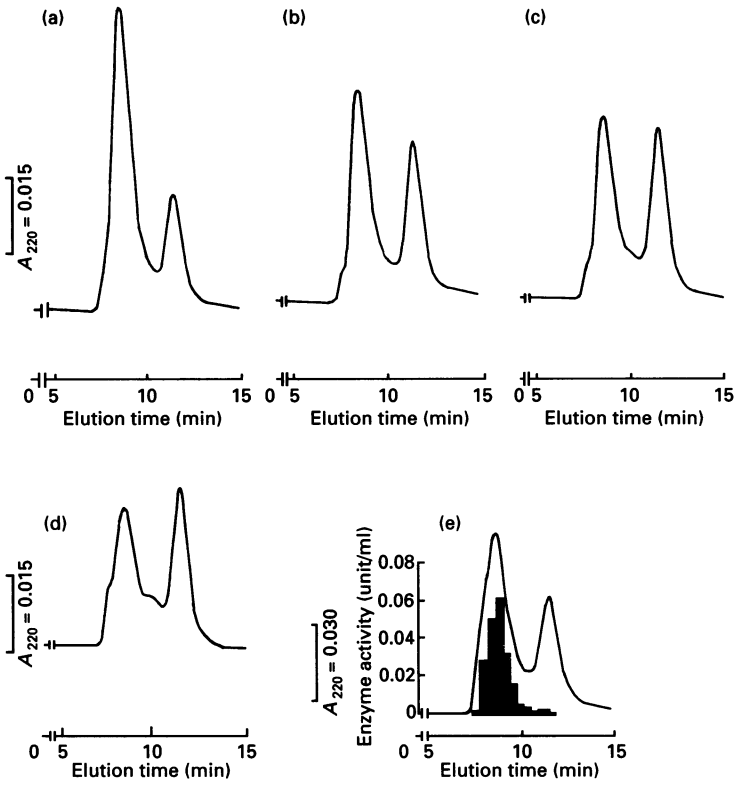


Figure 2 Elution profiles of diluted PC chromatographed on a TSK G3000 SW high-performance gel-filtration column

PC was diluted to 0.5 unit/ml in 0.1 M Tris/HCl, pH 7.2, containing 0.1 M KCl, and samples were analysed by h.p.l.c. as described in the Materials and methods section at (**a**) 20 min, (**b**) 60 min, (**c**) 100 min and (**d**) 240 min after dilution. (**e**) Elution profile of PC injected 100 min after dilution to 1 unit/ml, chromatographed under the same conditions as samples (a–d). The histogram under the protein profile represents the enzymic activity of 0.4 ml fractions of the eluted protein.

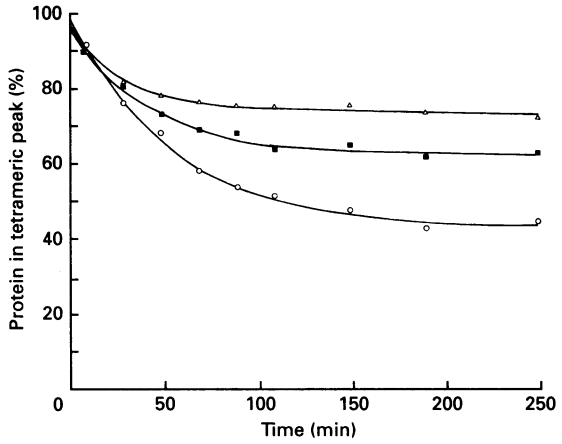


Figure 3 Time-dependent dissociation of PC after dilution

PC samples were diluted to 2 (△), 1 (■) or 0.5 (○) units/ml in 0.1 M Tris/HCl, pH 7.2, containing 0.1 M KCl, and the material was analysed by h.p.l.c. at various times after dilution, as described in the Materials and methods section. The protein remaining in the tetramer peak for each time point was calculated, and expressed as a percentage of the total area under the elution profile minus the estimated area under the aggregated protein peak. The lines were fitted as described in the legend to Figure 1.

Table 2 Parameters derived from fitting curves that describe first-order decay to a finite level of dissociation to data describing the time-dependent dissociation of the tetrameric enzyme on dilution

Initial enzyme concn. (units/ml)	Intercept at zero time (% of tetramer)	Final equilibrium level of dissociation (% of tetramer)	Pseudo-first-order rate constant (min^{-1})
2	97.5±2.9	73.3±0.7	0.033±0.005
1	97.0±2.3	61.7±0.7	0.023±0.002
0.5	100.7±2.6	42.3±1.0	0.018±0.001

second protein peak was eluted between the expected elution times for the dimeric and monomeric forms of PC and contained very little enzymic activity. As suggested by Khew-Goodall et al. (1991), this material may represent dimers and monomers in equilibrium, although we cannot rule out the possibility that either dimers or monomers are eluted anomalously. Thus, under the conditions of the assay, dimers and monomers were essentially inactive. By 240 min after dilution to 0.5 unit/ml, another species began to appear between the two main peaks. Whereas the elution time of this material corresponded to that expected for PC trimers, such molecules have not been observed either in the electron microscope (Mayer et al., 1980) or in sedimentation studies (Taylor et al., 1978), and therefore it is most likely to represent a non-specific aggregate.

The area under the tetramer peak was calculated from similar chromatographs for several enzyme concentrations at various times after dilution. A time-dependent dissociation of the

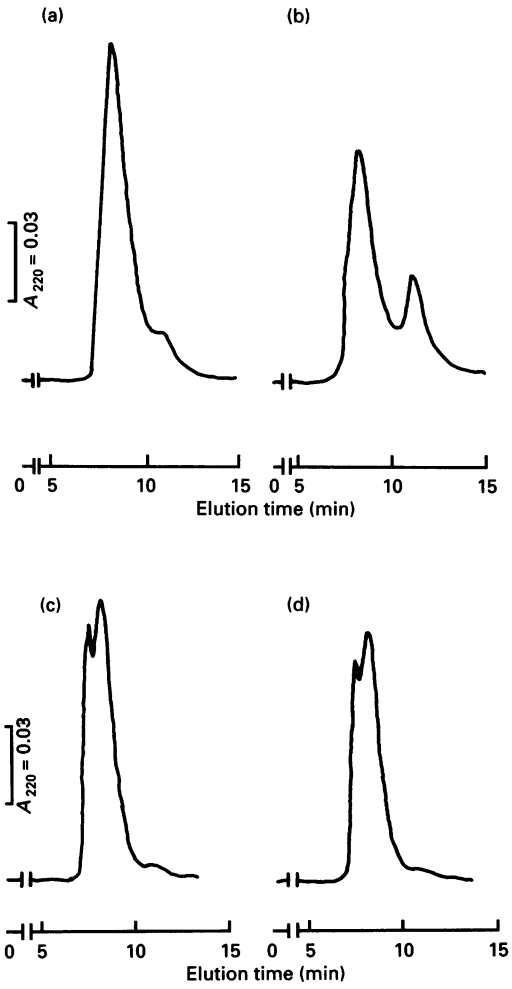


Figure 4 Effect of acetyl-CoA on dissociation of PC after dilution

PC was diluted to 1 unit/ml in 0.1 M Tris/HCl, pH 7.2, containing 0.1 M KCl with and without acetyl-CoA, and the molecular species were analysed by h.p.l.c. as described in the Materials and methods section. (**a**) 1 min after dilution without acetyl-CoA. (**b**) 40 min after dilution without acetyl-CoA. (**c**) 1 min after dilution into 0.1 mM acetyl-CoA. (**d**) 40 min after dilution into 0.1 mM acetyl-CoA. Samples (**c**) and (**d**) also contained 0.1 mM acetyl-CoA in the running buffer.

tetramer into dimers and monomers occurred at all concentrations of PC examined, such that the final degree of dissociation increased as the initial level of enzymic activity decreased (Figure 3). As with the inactivation process, the curves fitted to the data indicate that the dissociation process was first-order and monophasic. In addition, the ordinate intercept of 100% demonstrates that the effect of further dilution on the column was minimal. The parameters derived from the computer fits are given in Table 2. Unlike the inactivation process, the rate constants appear to vary with enzyme concentration.

When PC was subjected to h.p.l.c. immediately after dilution, the first protein peak was eluted slightly ahead of both the expected elution time of the PC tetramer and the peak of enzymic activity, indicating that an inactive species was present in the initial concentrated enzyme solution. Considering the purity of the PC sample, this higher-molecular-mass species must be an aggregated form of PC. From the calibration curve, the molecular mass was estimated to be 600–650 kDa, and the inactive material was eluted closely with the fraction selected in the h.p.l.c. purification of the starting material. The elution profiles of diluted PC samples which were used to calculate the data points shown in Figure 3 indicated the presence of aggregated material, which was seen as a leading shoulder of the tetramer peak (see, e.g., Figure 2d). At PC dilutions of 0.5 and 1 unit/ml, only a small increase in the amount of aggregated protein was observed during the 4 h time course (1.4% increase over 4 h at 0.5 unit/ml), whereas at 2 units/ml a more marked increase of 9% occurred over 4 h. As the overall shape of the aggregate peak is not clearly defined, the quantification of the aggregated protein is at best an approximation, and the calculation of the apparent first-order rate constants will be affected by the variable aggregation observed at the different enzyme concentrations. Thus the data in Table 2 are estimates, and caution should be exercised when drawing conclusions. However, even with this proviso, it seems likely that the rate-limiting step for the dissociation process is concentration-dependent. Since the rate constants derived from the activity data (Table 1) are of similar magnitude to those derived from the dissociation data (Table 2), the two processes appear to be closely related.

It is also evident that the final level for the loss of tetrameric structure is less than that for the loss of activity at all enzyme concentrations (cf. Figures 1 and 3). The presence of the inactive

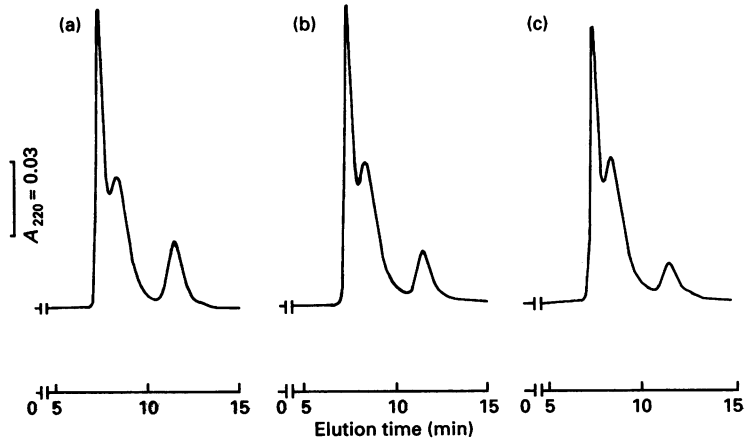


Figure 5 Reassociation of diluted PC in the presence of acetyl-CoA

PC was diluted to 1 unit/ml in 0.1 M Tris/HCl, pH 7.2, containing 0.1 M KCl. After 110 min, acetyl-CoA was added to a final concentration of 0.1 mM. The molecular species were analysed by h.p.l.c. at (**a**) 0 min, (**b**) 40 min and (**c**) 120 min after addition of acetyl-CoA, as described in the Materials and methods section, except that the running buffer also contained 0.1 mM acetyl-CoA.

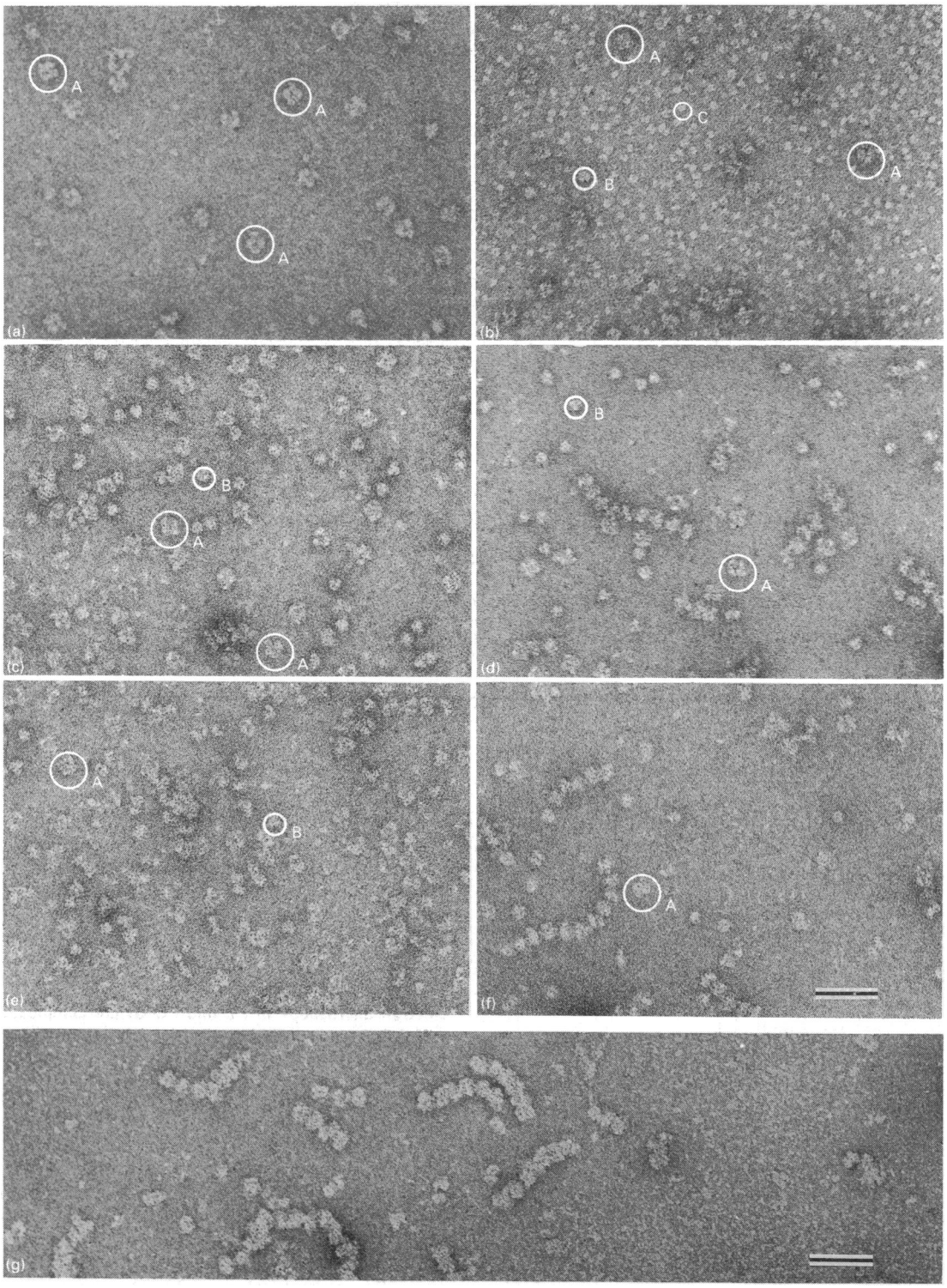


Figure 6 Electron micrographs of PC and avidin–PC complexes

(**a**)–(**f**) PC was incubated at 16 units/ml in the presence of 0.25 mM acetyl-CoA for 20 min, and then, where indicated, avidin was added at a ratio of 10:1 or 1:2, and the sample was incubated for a further 1 h. Samples were diluted in the presence or absence of acetyl-CoA and mounted for electron microscopy as described in the Materials and methods section. The final concentration of the mounted sample refers to PC concentration. (**a**) Incubation without avidin, and dilution into acetyl-CoA to 10 μg/ml. (**b**) Incubation with avidin at a ratio of 10:1 avidin:PC, and dilution into acetyl-CoA to 10 μg/ml. (**c**) Incubation without avidin, and dilution into acetyl-CoA to 40 μg/ml. (**d**) Incubation with avidin at a ratio of 1:2 avidin:PC, and dilution into acetyl-CoA to 40 μg/ml. (**e**) Incubation without avidin, and dilution without acetyl-CoA to 40 μg/ml. (**f**) Incubation with avidin at a ratio of 1:2 avidin:PC, and dilution without acetyl-CoA to 40 μg/ml. (**g**) PC was removed from sucrose storage buffer and incubated at 0.5 unit/ml with avidin at a ratio of 1:2 avidin:PC for 1 h, then diluted to 40 μg/ml in the absence of acetyl-CoA before mounting for electron microscopy. Key: A, tetrameric enzyme particles; B, glutamate dehydrogenase; C, free avidin particles. The bar represents 50 nm.

species that is eluted slightly ahead of the active tetramer could partially account for this difference. In addition, the formation of the species eluted between the tetramer peak and the dimer/monomer peak (Figure 2d) during the time course would contribute to the inaccuracy in the estimation of the proportion of protein in the tetrameric form.

Table 3 Changes in the quaternary structure of PC dependent on the influence of avidin and acetyl-CoA as revealed by electron microscopy

Row no.	Presence of avidin (avidin:PC)	Preincubation with acetyl-CoA*	Dilution with acetyl-CoA†	PC tetramer (single particle) (%)‡	Presence of specific aggregation of tetrameric PC (chain-like structure)	Presence of non-specific aggregates	Presence of broken particles
(a) 1	–	+	+	100	–	–	–
2	–	+	–	1	–	+	+
3	–	–	+	8	–	+	+
4	–	–	–	1	–	+	+
5	10:1	+	+	100	–	–	n.d.
6	10:1	+	–	23	–	+	n.d.
7	10:1	–	+	19	–	+	n.d.
8	10:1	–	–	1	–	+	n.d.
(b) 9	1:2	+	+	34	+	–	–
10	1:2	+	–	8	+	+	+
11	1:2	–	+	4	–	+	–
12	1:2	–	–	1	–	+	–

* The enzyme was incubated at 16 units/ml in the presence or absence of 0.25 mM acetyl-CoA in 0.1 M Tris/HCl/0.1 M KCl, pH 7.2, at room temperature for 15–20 min, after which avidin was added as required.

† The solutions of enzyme or avidin + enzyme were diluted to 0.12 unit/ml (**a**) or 0.5 unit/ml (**b**) with 0.1 M Tris/HCl/0.1 M KCl, pH 7.2, which, where indicated, contained 0.25 mM acetyl-CoA.

‡ Percentages were calculated as described in the Materials and methods section such that the number of tetrameric particles is expressed as a percentage of that seen when the sample was prepared in the presence of acetyl-CoA. The control value was determined separately for each set of conditions. The value for single intact tetramers in row 9 indicates that 34% of the total tetrameric particles observed were in this form, whereas the remaining 66% were in the chain-like structures. Abbreviation: n.d., not determined, since such particles were bound to the excess avidin.

Effect of acetyl-CoA on dissociation after dilution

Figure 4 shows the elution profiles of samples of chicken liver PC analysed by h.p.l.c. after dilution to 1 unit/ml in the presence and absence of acetyl-CoA. The appearance of the dimer/monomer peak was completely prevented in the presence of acetyl-CoA. Most of the protein was eluted in the tetramer/aggregate peak, and a minor dimer/monomer peak was observed which in fact became smaller as a result of prolonged incubation with acetyl-CoA. Assaying for enzymic activity across these h.p.l.c. profiles showed that in both cases the activity was eluted with the tetramer peak (cf. Figure 2e).

Re-activation and reassociation in the presence of acetyl-CoA

In order to examine further the effect of the allosteric activator during dilution inactivation, acetyl-CoA was added to PC 110 min after dilution to 1 unit/ml, i.e. when dilution inactivation was nearing completion. At various time intervals thereafter, samples were analysed by h.p.l.c. The area under the dimer/monomer peak decreased with time after the addition of acetyl-CoA (Figure 5). We estimate that the tetrameric peak increased by about 8% during the 120 min incubation, although the presence of the overlapping high-molecular-mass aggregate peak made accurate quantification difficult. By 120 min after the addition of acetyl-CoA, 38% of the dissociated material had reassociated, and the enzymic activity increased by 16%, from 0.55 ± 0.011 to 0.64 ± 0.013 unit/ml ($n = 4$). Thus dilution inactivation was at least partially reversible in terms of both reassociation of the tetrameric structure of the enzyme and its activity. The results indicate that either the re-activation of the enzyme was slower than the reassociation, or that some of the reassociated protein was inactive, as was the case with sheep PC (Khew-Goodall et al., 1991). The reassociation reported by these workers was more rapid than we have seen with the chicken enzyme. The re-activation of the dilution-inactivated enzyme on addition of acetyl-CoA is the first recorded observation of such a phenomenon. With both the liver and kidney PC from sheep, although acetyl-CoA protected against further inactivation, no recovery of activity was seen (Ashman et al., 1972; Khew-Goodall et al., 1991). Similarly, acetyl-CoA protected chicken PC against cold inactivation but did not cause any re-activation (Irias et al., 1969).

Electron microscopy of PC and PC–avidin complexes: effect of acetyl-CoA on the stability of the PC tetramer and its interaction with avidin

After preincubation at a high enzyme concentration in the presence of acetyl-CoA, PC was incubated with avidin at an avidin:PC ratio of 10:1 or 1:2. The samples were then diluted with or without acetyl-CoA and mounted for electron microscopy. As the representative micrographs in Figure 6 show, there were numerous well-preserved tetramers present when the enzyme was preincubated and diluted in the presence of acetyl-CoA (Figure 6a), and this situation was not altered by addition of avidin at 10:1 (Figure 6b). Quantitative analysis of the micrographs showed that at an avidin ratio of 10:1 essentially all the PC molecules were present as single tetramers (Table 3, rows 1 and 5). No aggregates or broken particles (e.g. PC dimers and monomers) were detected. As discussed previously (Johannssen et al., 1983), it was difficult to see the avidin bound to the tetrameric configuration of the enzyme. Samples diluted without acetyl-CoA did not retain the tetrameric structure (Figure 6e).

When the samples which had not been incubated with avidin were diluted in the absence of acetyl-CoA, only 1% of the enzyme molecules were detectable as tetramers (row 4). The material was present as monomers, dimers and non-specific aggregates. However, incubation with avidin at 10:1 resulted in

stabilization of the enzyme structure, such that 23% of the molecules remained tetrameric even without acetyl-CoA (row 6). Surprisingly, omitting the preincubation with acetyl-CoA before addition of avidin also had a significant detrimental effect on the stability of the tetramer (row 7), and, where dilution also occurred in the absence of acetyl-CoA, avidin was unable to preserve the tetrameric structure at all (row 8). It is apparent that preservation of the tetrameric PC structure was greatly enhanced by incubation of the enzyme with avidin at 10:1, and that both the preincubation and dilution with acetyl-CoA had a stabilizing effect.

When avidin was present at a ratio of 1:2 avidin:PC, and preincubation and dilution were performed in the presence of acetyl-CoA, chain-like structures composed of tetrameric PC molecules joined by avidin molecules were visible, along with single avidin–PC-tetramer complexes (Figure 6d) as previously described (Johannssen et al., 1983). The proportion of single tetramers was estimated to be 34% (Table 3, row 9), taking 100% as 350 tetramers/1500 cm^2. Thus we calculated that 66% of the total material was arranged in the chains. The corresponding control without avidin contained well-preserved tetramers which exhibited no flattening (Figure 6c). Although omitting acetyl-CoA during dilution did not affect the proportion of chains in the sample, the number of single avidin–PC tetramers was decreased (Figure 6f, and Table 3, row 10) and the tetramers had a flattened appearance (cf. Mayer et al., 1980). However, when preincubation with acetyl-CoA was omitted, the chain-like structures were completely absent (Table 3, rows 11 and 12; Attwood et al., 1986). Both these samples contained non-specific aggregates of protein which probably represent complexes between avidin and monomeric or dimeric PC molecules. Where avidin was added after dilution of the enzyme in the absence of acetyl-CoA, no specific reassociation of the tetramers was seen, and only smaller protein particles were visible (results not shown).

Figure 6g shows the effect of avidin (1:2) on a PC sample which was taken directly from the sucrose storage buffer and not treated with acetyl-CoA at any stage. Chain-like structures were present, along with single PC–avidin complexes in which the tetrameric structure was well preserved.

DISCUSSION

Whereas the h.p.l.c. analysis showed that acetyl-CoA preserved the tetrameric form almost completely on dilution, the procedure used for electron-microscopic examination resulted in significant loss of tetrameric structure even when dilution occurred in the presence of the activator (Table 3, row 3). Since the h.p.l.c. results are more closely consistent with the loss of activity (Figure 1), we consider these to be the more accurate in terms of quantification of dissociation. Electron microscopy involves the additional steps of the mounting procedure which may interact with prior treatments. However, the electron-microscopy data are internally consistent and reveal significant new features of the dissociation phenomenon, particularly in relation to the effects of avidin.

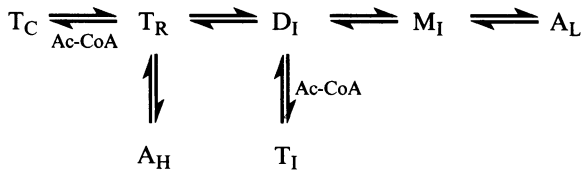
Previously, we have shown that one avidin molecule can act as a bridge between two subunits of a dimer within the tetrameric structure of PC (Johannssen et al., 1983). The results presented here (Table 3) show that at an avidin:PC ratio of 10:1 the stabilizing effect of avidin on PC structure after dilution was comparable with that of acetyl-CoA alone, whereas when PC molecules outnumbered avidin molecules, there was a much lower degree of stabilization of the isolated tetramer (i.e. those which were not part of the chains). This suggests the quaternary structure is stabilized when both dimers in the tetramer have their component monomers linked by avidin. A structure-stabilizing effect of avidin has also been observed in the enzyme isolated from *Aspergillus nidulans* (Osmani et al., 1984).

PC samples diluted for electron microscopy with and without acetyl-CoA show, respectively, compact and splayed tetrameric molecules (Mayer et al., 1980). Although this gross flattening or splaying of the tetramers may be caused largely by mounting for electron microscopy, it is apparent that in solution acetyl-CoA induces the tetramers to adopt a more compact conformation whereby avidin can bind the biotins from adjacent subunits, bridging the PC dimer. The results presented here suggest that two forms of the PC tetramer, a 'compact' and a 'relaxed' form, may be present over a range of protein concentrations, as indicated by the observation that the ability of avidin both to stabilize the tetramer on dilution and to form chains at the appropriate concentration were dependent on the preincubation with acetyl-CoA. A small but significant conformational change consistent with the formation of a more compact tetrameric structure has been observed in the presence of acetyl-CoA by measurement of u.v.-absorption spectra (Frey and Utter, 1977), a fluorescent probe (McGurk and Spivey, 1979) and in sedimentation studies (Taylor et al., 1978). It is possible that acetyl-CoA may cause the reassociation and re-activation reported here by altering an equilibrium between the two tetrameric forms in favour of the more compact form.

The presence of chains when PC was removed from storage buffer and incubated with avidin (1:2; Figure 6g) indicates that, in this case, the enzyme retained a compact structure in the absence of acetyl-CoA. The major component of the storage buffer is sucrose, and the protective effect of this substance against cold inactivation of PC has been known for some time (Irias et al., 1969). More generally, Lee and Timasheff (1981) have found that sucrose solutions raise the activation energy of unfolding of proteins, thus inducing the protein to remain in, or to adopt, a compact state where its contact with the solvent is minimized.

We suggest a scheme which describes the association states of PC as they are affected by concentration and acetyl-CoA. Scheme 1 is consistent with the results presented here and is based on the scheme of Khew-Goodall et al. (1991) for dilution inactivation in sheep liver PC. Unlike dilution inactivation of sheep PC, Scheme 1 indicates that there is no irreversible process occurring during the dilution-inactivation time course. With chicken PC, the dimers and monomers formed after dilution remain in equilibrium throughout. There is a low-molecular-mass aggregate formed from dissociated material, and a high-molecular-mass aggregate formed from tetramers. The high-molecular-mass aggregates which formed in both concentrated and dilute solutions may resemble the octameric form of PC detected by Taylor et al. (1978) in sedimentation studies. Acetyl-CoA shifts the equilibrium between T_C and T_R in favour of T_C. This accounts for the electron-microscopy observations and the re-activation produced by acetyl-CoA. Acetyl-CoA also promotes the formation of T_I, which allows for the observed reassociation in excess of re-activation.

In order to examine more closely the consequences of different reaction schemes and rate-limiting steps on the kinetics of the loss of enzymic activity, computer simulations of the loss of T_R at different initial concentrations were performed on four models based on Scheme 1 (obtainable from the author on request). Since the loss of activity was apparently first-order and did not depend on initial enzyme concentration, the first model to be simulated included an inactive tetramer intermediate between T_R and D_I (model 1). In the second model the rate of loss of T_R was governed by the formation of the equilibrium between T_R and D_I (model 2), whereas in the third model the loss of T_R was governed



Scheme 1

T, D and M represent tetrameric, dimeric and monomeric forms of PC. The subscripts C, R and I denote respectively compact, relaxed and catalytically inactive species. A_H and A_L denote high- and low-molecular-mass aggregates.

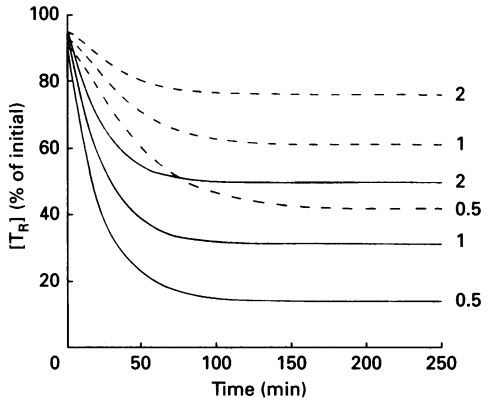
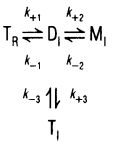


Figure 7 Computer simulation of the loss of tetramers and enzymic activity after dilution at different initial enzyme concentrations

Simulations were carried out with the simulation program KSIM (see the Materials and methods section) based on the mechanism described in Scheme 1, such that:



and initial $[D_I] = [M_I] = [T_I] = 0$ for all curves. Rate constants have arbitrary values, i.e. $k_{+1} = 10$, $k_{-1} = 500$; $k_{+2} = 0.2$, $k_{-2} = 0.004$; $k_{+3} = 0.15$, $k_{-3} = 0.04$; chosen so that $T_R \rightleftharpoons D_I$ is fast compared with $D_I \rightleftharpoons M_I$ and weighted in favour of T_R. Initial $[T_R]$ was, as indicated, 2, 1 or 0.5, and continuous or broken curves represent $[T_R]$ or $([T_R]+[T_I])$ respectively. The apparent first-order rate constants generated by this simulation are as follows

Initial $[T_R]$	Apparent first-order rate constant (min^{-1}) (loss of activity)	Apparent first-order rate constant (min^{-1}) (dissociation)
2	0.0427	0.0282
1	0.0433	0.0261
0.5	0.0454	0.0212

by the formation of the equilibrium between D_I and M_I (model 3). The model which gave simulated data closest in appearance to the actual data was in fact model 3. The simulations of models 1 and 2, where the rate of loss of T_R is more governed by first-order processes, gave apparent first-order rate constants that were most sensitive to initial $[T_R]$. The apparent first-order rate constants generated by simulation of model 3, where the formation of the equilibrium between D_I and M_I is slow in comparison to the formation of the equilibrium between T_R and D_I, were least sensitive to the initial $[T_R]$. Inclusion of T_I in model 3 yielded model 4, which in general gave similar results to the model 3 simulation: when $([T_R]+[T_I])$ was plotted, the loss of tetrameric protein was less than the loss of active tetramer as given by $[T_R]$ (Figure 7). Thus model 4, which is closest to the proposed Scheme 1, gives data that are similar to the experimental data (cf. Figures 1 and 2). The simulation of loss of tetrameric enzyme $([T_R]+[T_C])$ indicates an initial small logarithmic phase; however, this was not observed in the experimental data, as the first time point was after this phase. Although we cannot rule out other possible schemes, Scheme 1 seems to be the simplest that will explain the data from both kinetic and electron-microscopic experiments. In addition, the simulations of models 3 and 4 have indicated that there is very little accumulation of D_I, and hence imply that most of the dissociated material in the h.p.l.c. traces was monomer being eluted anomalously, possibly owing to partial unfolding.

Scheme 1 has many features in common with that proposed for cold inactivation in chicken liver PC (Irias et al., 1969; Taylor et al., 1978). Among these are the presence of an inactive tetramer, and the acetyl-CoA-induced formation of inactive tetramers and aggregates. It is possible therefore that dilution and cold inactivation involve similar processes.

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