A new spectrophotometric assay for citrate synthase and its use to assess the inhibitory effects of palmitoyl thioesters

Anthony J. ELSE,* Sarah J. BARNES, Michael J. DANSON† and P. David J. WEITZMAN

Department of Biochemistry, University of Bath, Bath BA2 7AY, U.K.

We have demonstrated that citrate synthase may be assayed by a simple, discontinuous, spectrophotometric procedure based on the measurement of oxaloacetate utilization with 2,4-dinitrophenylhydrazine. The assay is applicable both to the purified enzyme and to cell extracts, and has the advantage that it can be used in the presence of high concentrations of thiols and thioesters. We have used this new assay in part of our investigations into the inhibitory effects of palmitoyl thioesters on diverse citrate synthases. Both palmitoyl-CoA and palmitoyl thioglycollate inhibit citrate synthases from pig heart, *Bacillus megaterium* and *Escherichia coli*, the *E. coli* enzyme showing the greatest sensitivity to these effectors. With palmitoyl-CoA the extent of inhibition is time-dependent, but the enzymes can be protected from the effect by the substrates oxaloacetate and acetyl-CoA. Using the dinitrophenylhydrazine assay, we have shown that the thioester bond is essential for inhibition; that is, if the palmitoyl thioesters are cleaved to give a mixture of palmitate and a thiol compound, the inhibitions of pig heart and *B. megaterium* citrate synthases are eliminated and that of the *E. coli* enzyme is markedly decreased.

INTRODUCTION

Citrate synthase (EC 4.1.3.7) catalyses the reaction:

The enzyme is important in the control of the citric acid cycle, and its kinetic and regulatory properties have been found to correlate remarkably with the taxonomic status of the source organism (Weitzman & Danson, 1976; Weitzman, 1981; Danson *et al.*, 1985).

Several methods have been employed to assay citrate synthase. The most commonly used is the continuous spectrophotometric assay with 5,5'-dithiobis-(2-nitrobenzoate) (Nbs₂); this chromogenic reagent reacts with the thiol group of free CoA as it is produced in the formation of citrate (Srere *et al.*, 1963). Other assays for citrate synthase, also based on the measurement of CoA, use dichlorophenol-indophenol (Wieland *et al.*, 1964) or polarographic analysis (Weitzman, 1969b). Clearly, these procedures are inappropriate should it be necessary to assay citrate synthase in the presence of initial concentrations of thiol compounds. An alternative assay for citrate synthase relies on measurement of acetyl-CoA consumption by monitoring the decrease in A₂₃₂ (Srere & Kosicki, 1961); this assay can be used in the presence of thiol compounds, but suffers from the disadvantage that it is not readily applicable to cell extracts or when other u.v.-absorbing materials are present.

In the present paper we describe a discontinuous spectrophotometric assay for citrate synthase based on the measurement of oxaloacetate utilization with 2,4-dinitrophenylhydrazine (Dnph). This assay is unaffected by the presence of thiols and is also suitable for assaying citrate synthase in cell extracts.

We demonstrate the usefulness of this new assay by its application to a study of the inhibition of citrate synthases by palmitoyl-CoA. Palmitoyl-CoA is a powerful inhibitor

of pig heart citrate synthase (Wieland & Weiss, 1963; Tubbs, 1963), and inhibition has also been reported for the *Escherichia coli* enzyme (Srere, 1968). The physiological significance of this inhibition is uncertain (Srere, 1965; Hsu & Powell, 1975; Caggiano & Powell, 1979; Hansel & Powell, 1984). In eukaryotes, it has been suggested that inhibition of citrate synthase by palmitoyl-CoA is important in the regulation of fatty acid biosynthesis and in ketogenesis, and Hansel & Powell (1984) have provided evidence for a specific interaction between fatty acyl-CoA and pig heart citrate synthase. On the other hand, a number of observations (Srere, 1965) suggests that the inhibition is a result of non-specific detergent effects of fatty acyl-CoA compounds. In an attempt to clarify the situation, a comparative study was undertaken of the effects of palmitoyl-CoA, palmitoyl thioglycollate and of palmitate plus free thiol compounds on the citrate synthases of pig heart, *Bacillus megaterium* and *E. coli*.

EXPERIMENTAL

Materials

CoA, oxaloacetate and pig heart citrate synthase were from Boehringer, Mannheim, Germany; Nbs₂ and Dnph were from Sigma, Poole, Dorset, U.K. *Bacillus megaterium* D101 was from our culture collection (P.D.J.W.). *Escherichia coli* DB1002 (Bloxham *et al.*, 1983) was kindly provided by Dr. D. Bloxham, University of Southampton.

Citrate synthase assays

All assays were carried out at 25 °C. The assay using Dnph is described in the Results section. Citrate synthase was also assayed by the Nbs₂ method of Srere *et al.* (1963).

Abbreviations used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoate); Dnph, 2,4-dinitrophenylhydrazine.

* Present address: Laboratoire d'Enzymologie, CNRS, 91190 Gif-sur-Yvette, France

† To whom all correspondence should be addressed.

Fig. 1. Assay of purified *B. megaterium* citrate synthase with Dnph

B. megaterium citrate synthase [(○) 35 munits and (●) 70 munits, as assayed by the method of Srere *et al.* (1963)] was incubated at 25 °C with 0.2 mM-oxaloacetate and 0.15 mM-acetyl-CoA in 20 mM-Tris/HCl (pH 8.0)/1 mM-EDTA. At known time intervals, samples were withdrawn from the assay mixture and the concentration of oxaloacetate was determined with Dnph as described in the Results section.

Purification of citrate synthases

Pig heart citrate synthase (Boehringer) was used without further purification. *B. megaterium* citrate synthase was purified by the method of Robinson *et al.* (1983). *E. coli* citrate synthase was purified by the method of Weitzman (1969a).

Cell extracts

A cell extract of *E. coli* was prepared from an 18 h culture of the organism grown in L-broth (10 g of tryptone/l; 5 g of yeast extract/l; 5 g of NaCl/l) (Lennox, 1955). The cells were harvested by centrifugation (5000 g for 10 min at 4 °C), resuspended in 20 mM-Tris/HCl (pH 8.0)/1 mM-EDTA, and disrupted by sonication (Ultrasonics 180 W sonicator) for 1 min in 15 s bursts with cooling. Cell debris was removed by centrifugation (10000 g for 30 min at 4 °C), and the supernatant was used without further purification.

Preparation of palmitoyl-CoA and palmitoyl thioglycollate

Palmitoyl-CoA and palmitoyl thioglycollate were prepared by the method of Chase & Tubbs (1972). The concentration of palmitoyl-CoA was estimated from its A_{260} ($\epsilon_{260} = 16.4 \times 10^6 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$).

RESULTS

Reaction of oxaloacetate with 2,4-dinitrophenylhydrazine

Dnph in acid solution reacts with the ketone group of oxaloacetate to give a yellow 2,4-dinitrophenylhydrazone

derivative. It is difficult to measure this derivative spectrophotometrically, as acid solutions of Dnph are themselves yellow. However, if the solution is made alkaline after the derivative has been produced, then a brick-red compound is obtained, and this compound can be measured spectrophotometrically. In the spectrum of this derivative of oxaloacetate there is a peak at 450 nm. Measurements at this wavelength can therefore be used to estimate oxaloacetate concentration and thereby to assay citrate synthase. The other substrates and products of the citrate synthase reaction (acetyl-CoA, citrate and CoA) do not react with Dnph.

A 120 μl portion of 10 mM-oxaloacetate was added to 3 ml of 0.1 M-HCl containing 0.5 mM-Dnph and, at recorded times, 0.5 ml samples were withdrawn and added to 0.5 ml of 2 M-NaOH. The A_{450} of the resulting solution was measured with time. The reaction was essentially complete after 10 min, and the compound formed was relatively stable; that is, the value of A_{450} decreased by < 5% over a period of 30 min after the addition of NaOH. In the experiments that follow, a 10 min incubation period was used, and the absorbances were recorded 5 min after the addition of NaOH.

Variation of the final oxaloacetate concentration (i.e. the concentration after the addition of the alkali) up to 100 μM produced a linear response in the A_{450} . Assuming complete reaction, the results give a molar absorption coefficient of $12500 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for the derivative.

Assay of purified citrate synthase from *B. megaterium*

B. megaterium citrate synthase was incubated at 25 °C with 0.2 mM-oxaloacetate and 0.15 mM-acetyl-CoA in 20 mM-Tris/HCl (pH 8.0)/1 mM-EDTA. Samples (50 μl) were withdrawn every 1 min for 5 min and added to 0.45 ml of 0.5 mM-Dnph in 1 M-HCl; this solution was left for 10 min before the addition of 0.5 ml of 3 M-NaOH. After a further 5 min, the A_{450} was recorded (Fig. 1). The reactions were approximately linear over the periods recorded. Fig. 2(a) shows that the measured enzymic activity is linear with respect to enzyme concentration up to about 60 munits of enzyme/ml of the original reaction mixture. Similar data were obtained with the pure pig heart citrate synthase. The assay is insensitive to the presence of thiol reagents; thus, when 10 mM-2-mercaptoethanol was included in the assay of the pig heart enzyme, the measured enzymic activity was indistinguishable from that determined in the absence of the reagent.

Assay of citrate synthase in a cell extract of *E. coli*

It was demonstrated that this assay procedure could be used to assay citrate synthase in cell extracts of *E. coli*; the change in A_{450} was linear with time, and the measured enzymic activities were directly proportional to the volume of extract in the assay (Fig. 2b).

Inhibition of citrate synthases by palmitoyl-CoA

The effects of palmitoyl-CoA on citrate synthase activity were investigated by using the Nbs₂ assay of Srere *et al.* (1963) (Fig. 3). In all cases, the assays were initiated by the addition of enzyme to the otherwise complete reaction mixture; that is, there was no preincubation of enzyme with inhibitor. Extensive inhibition was observed with the enzyme from pig heart, *B. megaterium* and *E. coli*. The *B. megaterium* citrate synthase behaved in a manner identical with that

Fig. 2. Linearity of measured enzymic activity with respect to enzyme concentration in the Dnph assay method

(a) Purified *B. megaterium* citrate synthase and (b) citrate synthase in cell extracts of *E. coli* were assayed by the Dnph method as described in the legend to Fig. 1.

of the pig heart enzyme, 50% inhibition being observed at 2 μ M-palmitoyl-CoA; the *E. coli* enzyme showed the greatest sensitivity to this effector, 50% inhibition being observed at 0.3 μ M-palmitoyl-CoA. Dixon plots (not shown) curved upwards, suggesting either co-operative inhibition or the occurrence of more than one inhibitory effect. Increasing the palmitoyl-CoA concentration or decreasing the concentration of either substrate increased the degree of curvature.

As reported for the pig heart enzyme (Srere, 1965), incubation of all three citrate synthases with oxaloacetate before the addition of palmitoyl-CoA considerably decreased the observed inhibition compared with that found when the effector was added before the substrate. Acetyl-CoA also afforded protection, but less so than

Fig. 3. Inhibition of citrate synthases by palmitoyl-CoA

Citrate synthases from pig heart (●), *B. megaterium* (Δ) and *E. coli* (○) were assayed at 25 °C by the Nbs₂ method of Srere *et al.* (1963). Assays were initiated by the addition of enzyme.

Fig. 4. Inhibition of citrate synthases by palmitoyl thioglycollate

Citrate synthases from pig heart (●), *B. megaterium* (Δ) and *E. coli* (○) were assayed at 25 °C by the Nbs₂ method of Srere *et al.* (1963). Assays were initiated by the addition of enzyme.

oxaloacetate. Moreover, the longer the period of incubation before addition of substrates, the greater the observed inhibition. This time-dependent 'inhibition' was largely irreversible and, as stated above, for this reason the measurements of the inhibitions by palmitoyl-CoA were made without preincubation of enzyme and effector. Under these conditions of assay the inhibitions are reversible, although the existence of the irreversible inactivations by palmitoyl-CoA could have contributed to the non-linearity of the Dixon plots.

Table 1. Relief of inhibition by palmitoyl-CoA and palmitoyl thioglycollate on breakage of the thioester bond

Citrate synthase was assayed by using the Dnph method as described in the text. Activities are expressed as munits/assay. Values in parentheses are percentage activities with respect to the assay with no added palmitoyl thioester. Cleavage of thioester bonds was carried out as described in the text.

Addition to assay	Citrate synthase activity (munits/assay)		
	<i>E. coli</i>	<i>B. megaterium</i>	Pig heart
None	1.64 (100 %)	1.50 (100 %)	1.92 (100 %)
300 μ M-palmitoyl-CoA	0.01 (0 %)	0.01 (0 %)	0.01 (0.5 %)
Treated palmitoyl-CoA (thioester bond broken)	0.97 (59 %)	1.36 (91 %)	1.91 (99.5 %)
None	1.64 (100 %)	1.50 (100 %)	1.92 (100 %)
260 μ M-palmitoyl thioglycollate	0.01 (0 %)	0.13 (9 %)	0.08 (4 %)
Treated palmitoyl thioglycollate (thioester bond broken)	1.07 (65 %)	1.39 (93 %)	1.66 (86 %)

Inhibition of citrate synthases by palmitoyl thioglycollate

Palmitoyl thioglycollate was also found to be a powerful inhibitor of all three citrate synthases (Fig. 4); again, *E. coli* citrate synthase was the most sensitive, whereas the effects on *B. megaterium* and pig heart citrate synthases were almost identical. Though the behaviour of the citrate synthases in response to palmitoyl thioglycollate was similar to that in response to palmitoyl-CoA, it did differ in that the observed inhibition was not time-dependent.

Cleavage of thioester bonds of palmitoyl-CoA and palmitoyl thioglycollate

The thioester bond of palmitoyl-CoA can be cleaved by incubation for 90 min at pH 13 and 50 °C, and that of palmitoyl thioglycollate by a similar incubation at neutral pH. These cleavages result in the generation of free thiol groups, making the assay of citrate synthase in the presence of these compounds impossible by the Nbs₂ method. Therefore we have used the Dnph assay method to determine the effects of the cleaved palmitoyl-CoA and palmitoyl thioglycollate on the three citrate synthases.

The observations are presented in Table 1. As expected, similar inhibitions of the citrate synthases by palmitoyl-CoA and palmitoyl thioglycollate were found with the Dnph and Nbs₂ assays, adding further weight to the validity of the assay method reported in the present paper. Cleavage of the thioester bonds almost completely relieved the observed inhibitions of the pig heart and *B. megaterium* citrate synthases, and significantly decreased that of the *E. coli* enzyme. Consistent with these effects, we have found that 0.2 mM-palmitate alone had no effect on the citrate synthases of *B. megaterium* and pig heart, whereas similar concentrations caused approx. 25 % inhibition of the enzyme from *E. coli*.

DISCUSSION

One purpose of the present paper is to report a new assay for citrate synthase, based on the measurement of oxaloacetate utilization with Dnph. The assay has been developed and successfully tested with both unfrac-

tionated cell extracts and purified citrate synthase, and in the presence and absence of free thiol compounds. For most circumstances the assay is not intended to replace existing methods of measuring citrate synthase activity; however, it is considered that it will be the assay of choice when, for example, impure samples of citrate synthase are to be measured in the presence of thiol-containing molecules.

The usefulness of the Dnph method has been demonstrated with an investigation of the palmitoyl-CoA inhibition of a variety of citrate synthases, to compare the effectiveness of this inhibitor with respect to palmitoyl thioglycollate and free palmitate and CoA. The Nbs₂ assay was used initially to confirm previously reported data that palmitoyl-CoA is a powerful inhibitor of both pig heart and *E. coli* citrate synthases (Wieland & Weiss, 1963; Tubbs, 1963; Srere, 1968). In addition, we report, for the first time, that *Bacillus* citrate synthase (a dimeric enzyme, as is the protein from all eukaryotic sources) is inhibited to precisely the same degree as the pig heart enzyme. The CoA moiety of palmitoyl-CoA is not essential for inhibition, since palmitoyl thioglycollate inhibited the three citrate synthases, although higher concentrations of the inhibitor were required than with palmitoyl-CoA. Again, the *Bacillus* citrate synthase responded in a manner identical with that for the pig heart enzyme.

Using the Dnph method, we have confirmed the above observations with palmitoyl-CoA and palmitoyl thioglycollate. In addition, we have cleaved the thioester bond in both of these compounds and demonstrated that this treatment relieved the inhibitory effects on pig heart and *Bacillus* citrate synthases, and markedly decreased that on the *E. coli* enzyme. Thus, as reported for the eukaryotic enzyme (Wieland & Weiss, 1963), we conclude that the thioester bond is the feature essential to the inhibitory action of palmitoyl thioesters on both eukaryotic and bacterial citrate synthases.

The identical behaviour of the dimeric *Bacillus* and pig heart citrate synthases is noteworthy. It has been suggested that in eukaryotic systems palmitoyl-CoA inhibition of citrate synthase is important in the regulation of fatty acid biosynthesis and ketogenesis (Wieland & Weiss, 1963; Hansel & Powell, 1984). This

may indeed be a possibility, but what is demonstrated in the present paper is that the inhibitory effects are not peculiar to the eukaryotic citrate synthase, but are similarly observed with a bacterial enzyme of similar subunit structure and kinetic properties. An even greater effect is found with the hexameric bacterial citrate synthase of *E. coli*; part of this increased sensitivity may be due to a detergent effect of the fatty acid chain, since palmitate alone caused significant inhibition. It is considered that any rationalization of the observed inhibitions must be valid for both eukaryotic and prokaryotic systems, and that present explanations are weakened by their limitation to the eukaryotic case.

We thank the Science and Engineering Research Council for studentships to A.J.E. and S.J.B. We also thank Miss Sue O'Neil for technical assistance.

REFERENCES

- Bloxham, D. P., Herbert, C. J., Ner, S. S. & Drabble, W. T. (1983) *J. Gen. Microbiol.* **129**, 1889–1897
- Caggiano, A. V. & Powell, G. L. (1979) *J. Biol. Chem.* **254**, 2800–2806
- Chase, J. F. A. & Tubbs, P. K. (1972) *Biochem. J.* **129**, 55–65
- Danson, M. J., Black, S. C., Woodland, D. L. & Wood, P. A. (1985) *FEBS Lett.* **179**, 120–124
- Hansel, B. C. & Powell, G. L. (1984) *J. Biol. Chem.* **259**, 1423–1430
- Hsu, K. L. & Powell, G. L. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4729–4733
- Lennox, E. S. (1955) *Virology* **1**, 190–206
- Robinson, M. S., Danson, M. J. & Weitzman, P. D. J. (1983) *Biochem. J.* **213**, 53–59
- Srere, P. A. (1965) *Biochim. Biophys. Acta* **106**, 445–455
- Srere, P. A. (1968) in *Metabolic Roles of Citrate* (Goodwin, T. W., ed.), pp. 11–21, Academic Press, New York
- Srere, P. A. & Kosicki, G. W. (1961) *J. Biol. Chem.* **236**, 2557–2559
- Srere, P. A., Brazil, H. & Gonen, L. (1963) *Acta Chem. Scand.* **17**, S129–S134
- Tubbs, P. K. (1963) *Biochim. Biophys. Acta* **70**, 608–609
- Weitzman, P. D. J. (1969a) *Methods Enzymol.* **13**, 22–26
- Weitzman, P. D. J. (1969b) *Methods Enzymol.* **13**, 365–368
- Weitzman, P. D. J. (1981) *Adv. Microb. Physiol.* **22**, 185–244
- Weitzman, P. D. J. & Danson, M. J. (1976) *Curr. Top. Cell. Regul.* **10**, 161–204
- Wieland, O. & Weiss, L. (1963) *Biochem. Biophys. Res. Commun.* **13**, 26–31
- Wieland, O., Weiss, L. & Eger-Neufeldt, I. (1964) *Biochem. Z.* **339**, 501–513

Received 13 August 1987/24 November 1987; accepted 4 January 1988