Citrate synthase: an immunochemical investigation of interspecies diversity

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Rabbit antibodies have been raised to pig heart citrate synthase. Using purified IgG, competitive enzyme-linked immunoassays and assays of citrate synthase activity indicate the presence of antibodies to a number of antigenic sites on the enzyme, only some of which are essential for catalytic activity. From a comparison of citrate synthases from prokaryotic and eukaryotic organisms, the degree of interaction between antibody and enzyme was in the order: pig heart > pigeon breast > *Bacillus megaterium* > *Escherichia coli*. These findings are discussed in terms of the known interspecies diversity of the enzyme.

Citrate synthase *Immunochemistry* *Enzyme diversity*

1. INTRODUCTION

Citrate synthase possesses a diversity of subunit structure, catalytic activity and allosteric regulation that shows a strong correlation with the taxonomic status of the source organism [1,2]. The 'large', hexameric [3,4] citrate synthases, found exclusively in Gram-negative bacteria [5], are allosterically inhibited by NADH and, in the facultative anaerobes of this group, the enzyme is subject to additional inhibition by 2-oxoglutarate [6,7]. In contrast, Gram-positive bacteria and eukaryotes possess 'small' dimeric citrate synthases [8–10] which are isosterically inhibited by ATP but are insensitive to NADH and 2-oxoglutarate [1,2,11].

To begin an examination of this diversity of citrate synthases at the fine structural level, we have compared the enzyme from a wide range of sources using immunochemical methods. Antibodies have been raised to pig heart citrate synthase and the cross-reactivities between the enzymes from the bacterial and eukaryotic organisms examined using both competitive enzyme-linked immunoassays and assays of citrate synthase activity.

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2. EXPERIMENTAL

All chemicals used were of analytical grade. Pig heart citrate synthase was from Boehringer (Mannheim, FRG) and pigeon breast muscle citrate synthase was from Sigma (Poole, England). Citrate synthases from *E. coli and B. megaterium* were purified as described previously [4,8]. All 4 enzymes ran as single protein bands on SDS gel electrophoresis. Enzyme activity was assayed spectrophotometrically at 412 nm [12].

Rabbits were immunized with pig heart citrate synthase and IgG was purified from the serum by precipitation with ammonium sulphate (45% saturated) followed by ion-exchange chromatography using DEAE-cellulose. This IgG was used in all experiments and is referred to as IgG-anti-PHCS.

IgG-anti-PHCS was assayed by an ELISA method [13]. EIA cuvettes were coated for 3 h at 37°C with pig heart citrate synthase (1 μg/ml of 0.1 M carbonate buffer, pH 9.6). After washing with 0.15 M NaCl containing 0.05% Tween 20 (saline/

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Tween), antibody samples at suitable dilutions in saline/Tween were added and incubated for 2 h at 37°C. After further washing, the cuvettes were treated with alkaline phosphatase-goat anti-rabbit IgG for 18 h at 4°C. Finally, the cuvettes were washed and incubated with substrate solution (*p*-nitrophenyl phosphate, 1 mg/ml of 0.05 M carbonate buffer, pH 9.8). The reaction was stopped with 1.0 M NaOH and the absorbance measured at 405 nm.

A competitive assay, based on the ELISA method, was used in cross-reactivity studies: IgG-anti-PHCS was incubated for 1 h at 37°C with the appropriate antigen in solution and then transferred to an antigen-coated cuvette. Other details for the competitive assay were as described above.

To detect interaction of antibody with enzyme in free solution, purified citrate synthase was incubated with IgG-anti-PHCS for 5 min, and then sufficient immobilized protein A in the form of intact *Staphylococcus aureus* cells was added to bind all the IgG. The mixture was incubated for 10 min at 25°C and centrifuged to sediment the cells. Samples of supernatant were assayed for citrate synthase activity as described above.

3. RESULTS

3.1. *Characterization of antibodies to pig heart citrate synthase*

The ELISA method was used to detect antibodies to pig heart citrate synthase in rabbit serum and to confirm retention of antibody activity during purification of IgG-anti-PHCS. The competitive ELISA detected 1–100 μg pure enzyme/ml whereas similar concentrations of albumin and myoglobin did not inhibit binding of antibody to solid-phase antigen (fig.1).

3.2. *Species cross-reactivity by competitive ELISA*

Competitive ELISA with pigeon citrate synthase as solid phase antigen was used to compare the effects of different competing purified citrate synthase antigens in the fluid phase (fig.2). Competition with solid phase antigen for binding to IgG-anti-PHCS was seen with citrate synthases in the order pig > pigeon > *B. megaterium*. The *E. coli* enzyme showed no significant interaction in this assay.

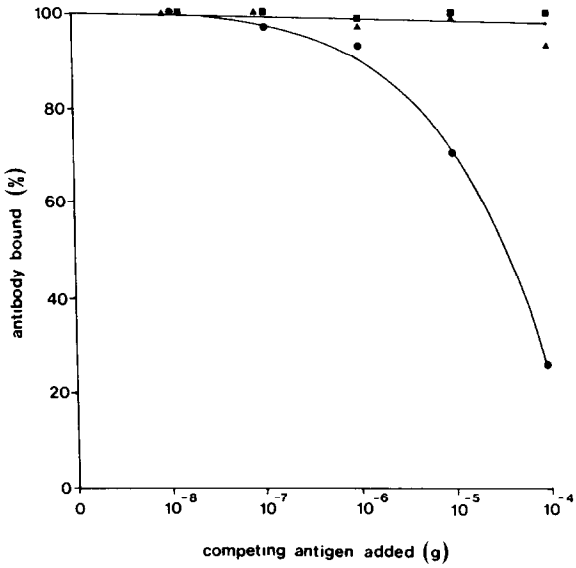

Fig.1. Inhibition of antibody binding to solid-phase antigen in the competitive ELISA system by addition of purified pig heart citrate synthase. Antiserum at standard dilution (1/1000) was incubated for 1 h at 37°C with varying amounts of pig heart citrate synthase (●), albumin (▲), or myoglobin (■), and then transferred to a cuvette coated with pig heart citrate synthase. Binding of antibody to the solid-phase antigen was assayed using alkaline phosphatase-goat anti-rabbit IgG, as described in section 2.

3.3. *Effects of antibody on citrate synthase activity*

IgG-anti-PHCS was found to inhibit citrate synthase enzymic activity, again in the order pig > pigeon > *B. megaterium* (fig.3). Normal rabbit IgG produced no inhibition. A small stimulation of the activity of the *E. coli* enzyme was observed, but its response to the allosteric effectors, NADH and 2-oxoglutarate, remained unaffected. IgG-anti-PHCS also inhibited citrate synthase in crude extracts of pig heart, liver, kidney and brain.

To detect binding of antibody to non-essential sites on the enzyme, citrate synthase-antibody immune complexes were removed with Protein A (see section 2). The amount of antibody required to produce a given level of inhibition of pig heart citrate synthase was reduced by this Protein A treatment (fig.4), suggesting that IgG-anti-PHCS contains antibodies to a number of antigenic sites on the citrate synthase molecule, only some of

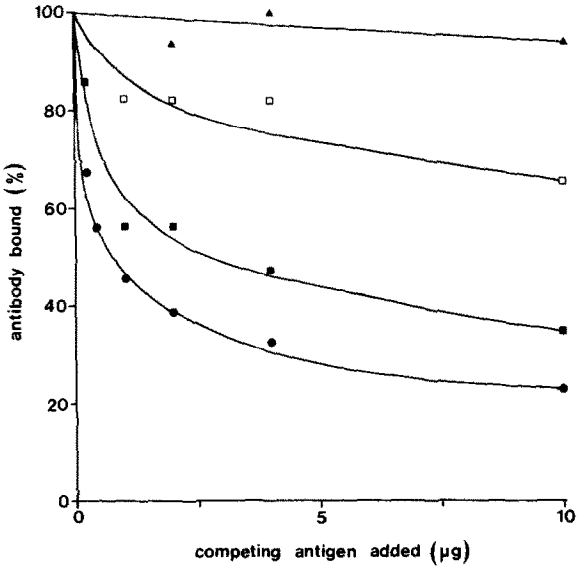

Fig.2. Inhibition of antibody binding to solid-phase antigen in the competitive ELISA by citrate synthases from various species. The antibody was incubated with varying amounts of citrate synthase from pig heart (●), pigeon breast (■), *B. megaterium* (□), or *E. coli* (▲) prior to transfer to cuvettes coated with pigeon breast citrate synthase. Binding of antibody to the solid-phase antigen was assayed using alkaline phosphatase-goat anti-rabbit IgG, as described in section 2.

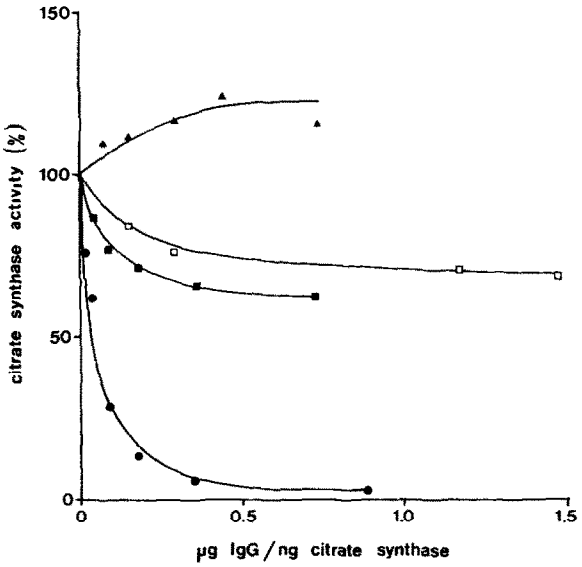

Fig.3. Effect of IgG-anti-PHCS on the enzymic activity of citrate synthase. Purified citrate synthase from pig heart (●), pigeon breast (■), *B. megaterium* (□), or *E. coli* (▲) was incubated with varying amounts of IgG-anti-PHCS and then assayed for residual citrate synthase activity as described in section 2.

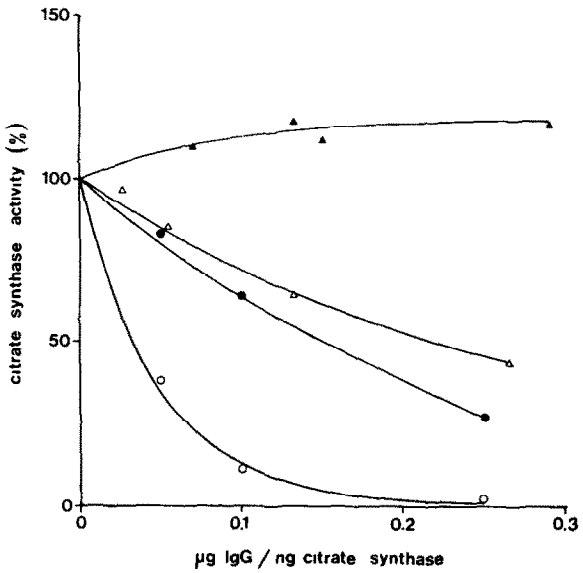

Fig.4. Inhibition of citrate synthase activity by IgG-anti-PHCS in the presence and absence of *S. aureus* protein A. Purified citrate synthase from pig heart or *E. coli* was incubated with varying amounts of IgG-anti-PHCS. Residual citrate synthase activity was then assayed as described in section 2 before and after removal of antibody-enzyme complexes using protein A. Pig heart citrate synthase before (●) and after (○) protein A: *E. coli* citrate synthase before (▲) and after (△) protein A.

which are essential for enzymic activity. Similarly, binding of antibody to *E. coli* citrate synthase was detected by the use of Protein A (fig.4) even though no inhibition of activity was observed (fig.3).

4. DISCUSSION

This paper represents the first report of an immunochemical comparison of citrate synthases from a wide range of organisms, and its significance stems from the established patterns of structural and regulatory diversity displayed by this enzyme [1,2].

With IgG purified from a rabbit polyclonal antiserum to pig heart citrate synthase, we have detected antibody activity against the enzyme using direct and competitive ELISA methods and through enzymic assays in the presence and absence of Protein A. These same methods were then used to compare citrate synthases from prokaryotic and eukaryotic organisms and the degree of interaction

between antibody and enzyme was found to be in the order: pig heart > pigeon breast > *B. megaterium* > *E. coli*.

The data establish several important points. The different types of citrate synthase do share structural similarities in that they appear to possess a number of common antigenic determinants. Alignment of the sequences of the *E. coli* and pig heart enzymes [14,15] shows regions of homology throughout the sequence, including amino acids implicated in the catalytic mechanism of the pig heart citrate synthase. Surprisingly, however, antibodies inhibiting enzymic activity of the pig enzyme did not inhibit that from *E. coli*. Virtually no structural data are available for citrate synthases from Gram-positive bacteria. The interaction of IgG-anti-PHCS was more pronounced with *Bacillus* citrate synthase than with the *E. coli* enzyme, suggesting that the citrate synthases from the Gram-positive bacterium and the eukaryote may be structurally close, not only in oligomeric and regulatory features [1,2] but also perhaps in sequence and/or conformation. Such observations add to the accumulating observed similarities between mitochondrial and Gram-positive bacterial citric acid cycle enzymes [1], similarities which should be borne in mind when considering organelle evolution.

Finally, it is felt that the results presented here using polyclonal antisera confirm the feasibility of investigating the structural diversity of citrate synthase using immunological methods. We have tested a number of different methods to detect antibody-enzyme interaction and the ability to do this in impure preparations will be essential to any immunochemical comparisons between enzymes from a large number of different organisms. With this basis, future work will involve a monoclonal antibody system which will enable us to define and identify on the different enzymes regions of common sequence and conformation and to investigate their relationship with enzymic activity and regulation.

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REFERENCES

[1] Weitzman, P.D.J. (1981) Adv. Micro. Physiol. 22, 185–244.
[2] Weitzman, P.D.J. and Danson, M.J. (1976) Curr. Top. Cell Regul 10, 161–204.
[3] Tong, E.K. and Duckworth, H.W. (1975) Biochemistry 14, 235–241.
[4] Robinson, M.S., Easom, R.A., Danson, M.J. and Weitzman, P.D.J. (1983) FEBS Lett. 154, 51–54.
[5] Weitzman, P.D.J. and Jones, D. (1968) Nature 219, 270–272.
[6] Wright, J.A., Maeba, P. and Sanwal, B.D. (1967) Biochem. Biophys. Res. Commun. 29, 34–38.
[7] Weitzman, P.D.J. and Dunmore, P. (1969) FEBS Lett. 3, 265–267.
[8] Robinson, M.S., Danson, M.J. and Weitzman, P.D.J. (1983) Biochem. J. 213, 53–59.
[9] Wu, J.Y. and Yang, J.T. (1970) J. Biol. Chem. 245, 212–218.
[10] Singh, M., Brooks, G.C. and Srere, P.A. (1970) J. Biol. Chem. 245, 4636–4640.
[11] Harford, S. and Weitzman, P.D.J. (1975) Biochem. J. 151, 455–458.
[12] Srere, P.A., Brazil, H. and Gonen, L. (1963) Acta Chem. Scand. 17, S129–S134.
[13] Engvall, E. and Perlmann, P. (1972) J. Immunol. 109, 129–135.
[14] Ner, S.S., Bhayana, V., Bell, A.W., Giles, I.G., Duckworth, H.W. and Bloxham, D.P. (1983) Biochemistry 22, 5243–5249.
[15] Bhayana, V. and Duckworth, H.W. (1984) Biochemistry 23, 2900–2905.