DESENSITIZATION OF PYRUVATE CARBOXYLASE AGAINST ACETYL CoA STIMULATION BY CHEMICAL MODIFICATION†

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Received February 21,1973

Sheep kidney pyruvate carboxylase has been desensitized against its allosteric effector, acetyl CoA, by limited covalent modification with trinitrobenzene sulphonic acid.

Trinitrophenylation of the enzyme resulted in a strong inhibition of the rate of the acetyl CoA-stimulated pyruvate carboxylation and enhancement of the rate of the acetyl CoA-independent reaction. A good correlation was found between the requirement for acetyl CoA of the exchange reactions catalysed by the enzyme and the extent of their inhibition by trinitrobenzene sulphonic acid modification.

Spectrophotometric data indicated that one to two lysyl residues per monomer were trinitrophenylated. Modification had only a slight effect on the sedimentation properties of the enzyme.

Pyruvate carboxylase (pyruvate:CO_2 ligase (ADP) EC.6.4.1.1.) isolated from sheep kidney cortical mitochondria is strongly activated by the allosteric effector, acetyl CoA. This compound decreases the apparent K_m values for HCO_3^- and pyruvate but not for $MgATP^{2-}$ and increases the V_{max} (1).

Earlier studies in this laboratory showed that the enzyme possesses a lysine residue whose integrity is essential for full enzymic activity (2). Modification of the enzyme with amino group reagents such as FDNB, TNBS and potassium cyanate resulted in a loss of catalytic activity. Acetyl CoA protected against the inhibitory action of these amino group reagents and

†This work was supported by Grant 65/15780 from the Australian Research Grants Committee.

Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene: TNBS, 2,4,6-trinitrobenzene sulphonic acid; TNP-, trinitrophenyl-; DNP-, dinitrophenyl-.

it was suggested (2) that the ε-amino group of the lysine may be involved in the enzyme-acetyl CoA interaction.

In the studies reported here, evidence is presented showing that the chemical modification of the lysyl group using TNBS desensitized the enzyme against acetyl CoA stimulation.

MATERIALS AND METHODS

All pyruvate carboxylase assay procedures, materials and enzyme preparations were as previously described (1). For kinetic experiments, the specific activity of the enzyme was 12 to 16 but for physical studies the enzyme was further purified by Sephadex G-200 chromatography to a specific activity of 21 to 23.

For chemical modification experiments, the enzyme was equilibrated with 0.05 M tris (Cl^-), pH 8.4 containing 0.1 M KCl, by gel filtration using Sephadex G-25, and then incubated at 0.5 to 4.5 mg of protein/ml with TNBS (1.3×10^{-4} M) at 25°. Although tris contains a primary amino group, negligible reaction occurred during the incubation period. At various time intervals, samples were removed and the modification reaction was quenched by adding to a lysine solution (50 mM, final concentration). For spectral and ultracentrifugation studies, the modified enzyme was separated from the quenching agent and by-products of the reaction by gel filtration using Sephadex G-25.

Acetyl CoA deacylase activity was measured at pH 8.4 in the presence of all the components of the carboxylation reaction using the DTNB titration method described by Scrutton and Utter (3). The pyruvate:oxaloacetate exchange reaction assay contained (in micromoles) in a final volume of 0.5 ml; tris (Cl^-), pH 8.4, 50; pyruvate, 1; oxaloacetate, 1, and enzyme. Five min after the addition of the enzyme, the reaction was started by adding 1 μC

of carrier-free 2[^{14}C]pyruvate. The reaction was stopped by adding 1 unit of avidin. Pyruvate and oxaloacetate were then transaminated to alanine and aspartate and separated by high voltage paper electrophoresis and their radioactivity determined. The orthophosphate:ATP exchange assay conditions were similar to those described by Scrutton *et al.* (4) except that the reaction was stopped by adding 0.1 ml of 5 N formic acid and the phosphate compounds were separated by the method of Gilliland *et al.*, (5) and their radioactivity determined.

RESULTS AND DISCUSSION

The desensitization of pyruvate carboxylase by TNBS against acetyl CoA stimulation is clearly demonstrated in Fig. 1 where the effect of trinitrophenylation on both the acetyl CoA-dependent and acetyl CoA-independent reactions is shown†. It can be seen that although the acetyl CoA-dependent reaction was inhibited by the chemical modification, the rate of the acetyl CoA-independent reaction increased substantially.

Sheep kidney pyruvate carboxylase is similar in many respects to the enzymes isolated from chicken and rat liver mitochondria which catalyse exchange reactions between ^{32}P-orthophosphate and ATP and between ^{14}C-pyruvate and oxaloacetate (4,6,7) and deacylate acetyl CoA (3). Since acetyl CoA is required for the ^{32}P-orthophosphate:ATP exchange reaction, stimulates the ^{14}C-pyruvate:oxaloacetate exchange and is the substrate for the deacylase activity, the effect of TNBS modification on these activities was investigated. The results are shown in Fig. 2 and it can be seen that the ^{32}P-orthophosphate:

†A similar observation has been made using enzyme isolated from rat liver (private communication, Dr. M.C. Scrutton).

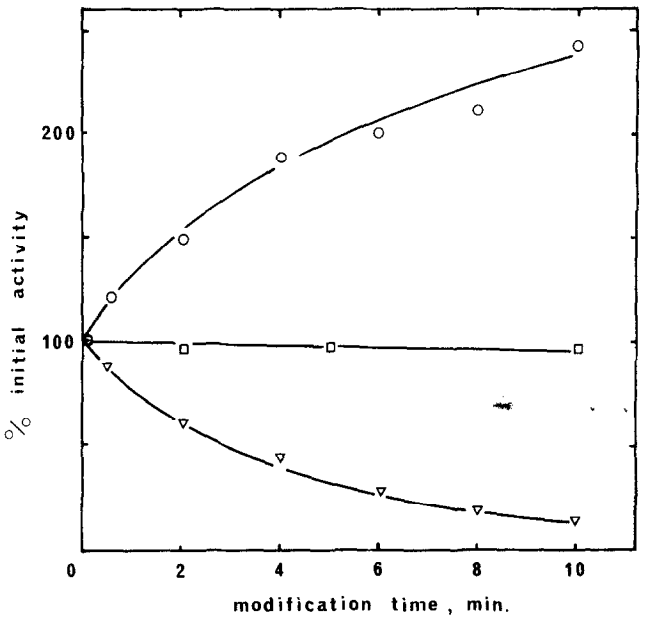

Fig. 1. The effect of trinitrophenylation on pyruvate carboxylase activity.

At the time intervals indicated, samples were withdrawn and after quenching the modification reaction, the acetyl CoA-dependent and acetyl CoA-independent pyruvate carboxylase activities were determined.

▽ —— ▽ , acetyl CoA-dependent activity; □ —— □ , control, i.e., incubated without TNBS and measured as the acetyl CoA-dependent activity; O —— O , acetyl CoA-independent activity.

ATP exchange reaction was inhibited to the same extent as the overall acetyl CoA-dependent reaction while the pyruvate:oxaloacetate exchange reaction (in the absence of acetyl CoA) and the deacylase activity were only slightly reduced.

The rate of the pyruvate:oxaloacetate exchange catalysed by the sheep kidney and rat liver enzymes is stimulated more than two-fold by acetyl CoA (6,7). The data presented in Fig. 3 show that the acetyl CoA stimulation was completely removed by trinitrophenylation while the basal rate of the exchange reaction was only slightly reduced.

The failure of TNBS modification to inhibit the deacylase activity implies that acetyl CoA can bind to the chemically

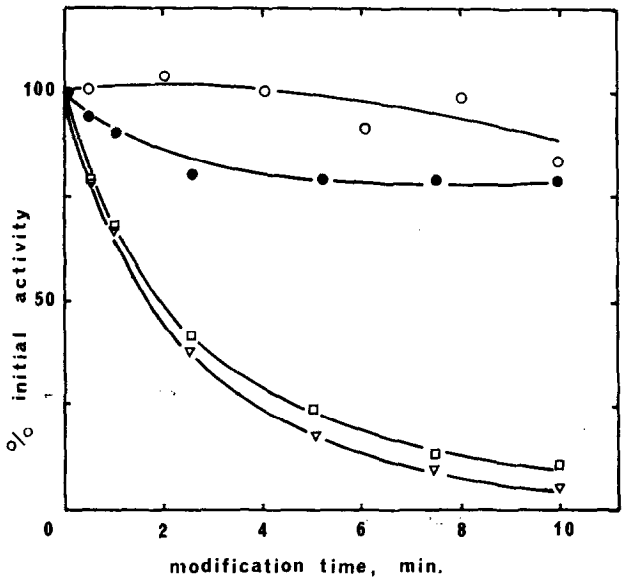

Fig. 2. The effect of trinitrophenylation on the exchange reactions and the deacylase activity.

▽ —— ▽, acetyl CoA-dependent activity; O —— O, deacylase activity; ● —— ●, pyruvate:oxaloacetate exchange activity; □ —— □, orthophosphate:ATP exchange activity.

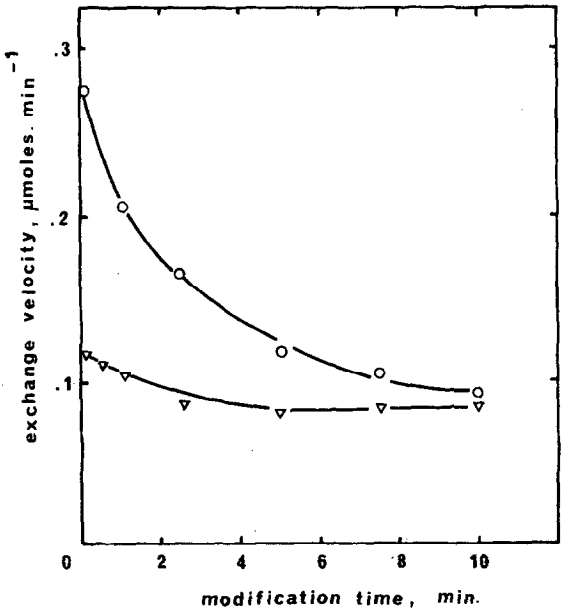

Fig. 3. Desensitization of the acetyl CoA stimulation of the pyruvate:oxaloacetate exchange reaction.

O——O, the exchange reaction carried out in the presence of 0.25 mM acetyl CoA; ▽ ——▽, the exchange carried out in the absence of acetyl CoA.

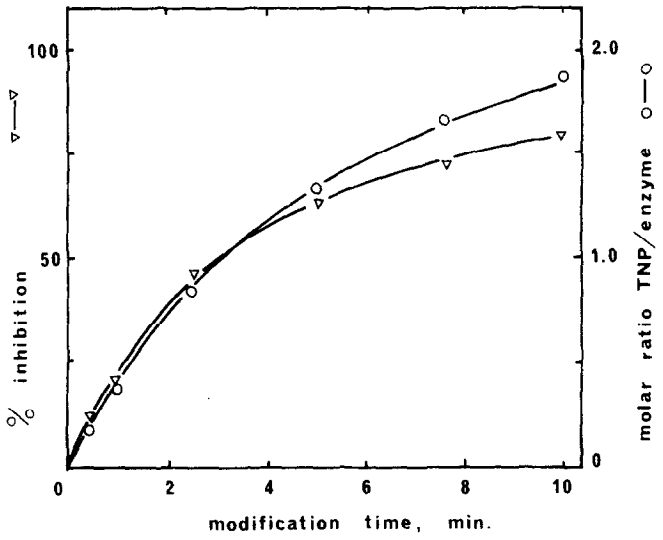

Fig. 4. Correlation between inhibition of the acetyl CoA stimulation of the pyruvate carboxylase reaction and triphenylation of the amino acid residues in the protein.

▽ —— ▽ , acetyl CoA-dependent pyruvate carboxylase activity; O —— O , molar ratio of TNP-derivative to enzyme monomer concentration.

modified enzyme. Therefore, the lysyl residue cannot be involved in binding acetyl CoA, but chemical modification must prevent the correct conformational change occurring when the allosteric effector binds. Since acetyl CoA protects the enzyme against TNBS modification it follows that the reactive lysyl residue is exposed in the absence of acetyl CoA but inaccessible in the acetyl CoA-activated conformation. Alternatively, the deacylase activity may not be a function of pyruvate carboxylase or it may be completely unrelated to the allosteric activation.

Although TNBS is known to react with thiol as well as primary amino groups (8,9) two lines of evidence support the previous finding (2) that the modifiable residue involved in acetyl CoA activation is lysine, (i) the trinitrophenylated enzyme has a spectrum characteristic of an amino-TNP derivative with absorption maxima at 345 and 420 nm (10), (ii) the TNP-enzyme

was not thiolysed by 2-mercaptoethanol. It has been shown that 2-mercaptoethanol removes DNP-moieties from amino acids, peptides (11) and proteins (12). We have found that TNP-derivatives behave similarly; S-TNP-cysteine but not ε-TNP-lysine is thiolysed by 2-mercaptoethanol.

Using a kinetic approach, Keech and Farrant (2) concluded that modification of one lysine residue per active site was involved in the inactivation process. However, this approach gave no indication of the total number of groups modified. Measurement of the increase in the absorbance at 367 nm due to formation of the TNP-derivative, and using a molar absorption coefficient of $1.05 \times 10^4\ M^{-1}$ for the TNP-derivative at this wavelength (13,14), showed that when the catalytic activity was reduced by 80%, the enzyme contained almost two TNP groups per monomer (M.Wt. 125,000) (Fig. 4). This value is most likely to be erroneously high since it is based on spectrophotometric protein determinations (15) and it has been shown for pyruvate carboxylases isolated from other sources that this method overestimates the true values by a factor varying between 1.43 and 2.0 (16,17, 18).

Analytical ultracentrifugation of the modified enzyme revealed that modification did not cause gross changes in the quaternary structure although some aggregation did occur. In a sample of enzyme which was 80% inactivated in the acetyl CoA-stimulated reaction, 20% of the protein (based on peak areas) existed as a 20S species while the remainder sedimented as a 15S species indistinguishable from the native enzyme. No dissociation to 7S monomers was observed.

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