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PYRUVATE CARBOXYLASE

EFFECT OF THE CHEMICAL MODIFICATION OF THE SULFHYDRYL GROUPS ON THE ACTIVITY AND QUATERNARY STRUCTURE*

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SUMMARY

1. Sulfhydryl reagents cause a profound effect on both the enzymatic activity and quaternary structure of pyruvate carboxylase (EC 6.4.1.1) from chicken liver (molecular weight of the tetramer 655 000, and about 55 sulfhydryl groups per molecule). At a molar ratio of inhibitor to enzyme (tetramer) equal to 36, *p*-hydroxymercuribenzoate (PHMB) or 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) causes a complete inactivation of the enzyme within 1 h. Iodoacetate and cystine inactivate the enzyme more slowly, and at relatively high concentrations give pseudo-first-order kinetics of inactivation. In contrast, iodoacetamide and *N*-ethylmaleimide inactivate the enzyme in two stages. The first phase is accompanied by a loss of activity of 35% with iodoacetamide and 75% with *N*-ethylmaleimide; the second phase results in complete inactivation in both cases.

2. The inactivation by PHMB, DTNB or iodoacetate is accompanied by a partial dissociation of the tetramers to monomers, and an aggregation to high-molecular-weight species. The enzyme inactivated by cystine shows only a conversion of tetramers to monomers. During the first stage of inactivation by *N*-ethylmaleimide, the enzyme retains its original sedimentation pattern, while during the second there is a formation of high-molecular-weight aggregates. These diverse effects are tentatively correlated with the nature of the different sulfhydryl reagents.

3. The inactivation by PHMB, DTNB or cystine can be partially reversed by thiols. With PHMB this reactivation was shown to be accompanied by a recovery of the original sedimentation pattern.

INTRODUCTION

The quaternary structure of pyruvate carboxylase (EC 6.4.1.1) from chicken

Abbreviations: PHMB, *p*-hydroxymercuribenzoate; DTNB, 5,5'-dithiobis-(2-nitrobenzoate); DTE, 2,3-dihydroxy-1,4-dithiolbutane.

* This paper is the 13th report in a series of studies on this enzyme.

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liver has been studied in detail in recent papers[1,2]. The enzyme has a molecular weight of about 655 000 and appears, in the electron microscope, as a tetramer formed by spherical monomers located in the corners of a square. Purified preparations of the enzyme contain a main tetrameric component ($s^0_{20,w}$ = 14.8 S) that seems to include the active species and a minor monomeric component ($s^0_{20,w}$ = 6.75 S)[1]. The enzyme is inactivated at 0–5° and shows dissociation from the 15-S to the 7-S component in the ultracentrifuge[1–3]. Electron microscopic examinations have shown that this shift is accompanied by a loss of tetrameric structure[2]. Both processes, inactivation and dissociation, are partially reversed upon rewarming[1–3].

Earlier work indicated the presence of about 55 sulfhydryl groups per tetrameric molecule and inactivation by certain thiol reagents[1,4]. The present paper reports a comparative study of the effects of different thiol reagents on the activity and quaternary structure of pyruvate carboxylase and describes the reversal by thiols of the effects produced by some of the sulfhydryl reagents. An abstract of some of the content of this paper has appeared previously[5].

MATERIALS AND METHODS

Chicken liver pyruvate carboxylase was prepared as described by SCRUTTON *et al.*[6]. Preparations purified through state 5 or 6 were used. In a few cases, preparations purified through state 5 were subjected to chromatography on a column of DEAE-Sephadex A50 using an $(NH_4)_2SO_4$ gradient for the elution (M. C. SCRUTTON, personal communication). The enzyme was stored in the cold at high protein concentrations (about 20 mg/ml) in 1.6 M sucrose, 0.1 M Tris–H_2SO_4 (pH 6.7), and 0.06 M $(NH_4)_2SO_4$. The experiments reported here were carried out with preparations of pyruvate carboxylase that had been equilibrated with 0.1 M potassium phosphate (pH 7.2), containing 60 mM $(NH_4)_2SO_4$, and 10 mM EDTA, by passage through a Sephadex G-25 column.

Pyruvate carboxylase was assayed spectrophotometrically in the direction of CO_2 fixation with the coupled malate dehydrogenase system. The conditions of the assay were as those described previously[6], except that the buffer used was Tris–H_2SO_4 instead of Tris–HCl. Specific activities are expressed as micromoles of oxalacetate formed per min per mg of protein at 25°. Protein was estimated spectrophotometrically by the method of WARBURG AND CHRISTIAN[7]. All incubations were carried out at room temperature.

Acetyl-CoA was prepared from coenzyme A (P-L Laboratories) and acetic anhydride as described by STADTMAN[8]. After exhaustive extraction with ether at pH 2.0, the acetyl-CoA solution was neutralized to pH 5.0. Acetyl-CoA concentrations were determined using citrate synthase as described by OCHOA[9]. Sigma Trizma base (Tris) was recrystallized from 1 mM EDTA in 85% ethanol, as described by SUTHERLAND AND WOSILAIT[10]. $(NH_4)_2SO_4$ was recrystallized twice from 1 mM EDTA, and 4,4′-dithiodipyridine was a gift from Dr. W. W. Cleland. Malate dehydrogenase was obtained from C. F. Boehringer. Other chemicals were reagent grade materials obtained from commercial sources.

Ultracentrifugal examinations were carried out in a Beckman Spinco Analytical Ultracentrifuge (Model E), using the AN-E rotor, 30-mm double-sector cells and at temperatures near 21°. Usually, the preparation for study was placed in a cell with

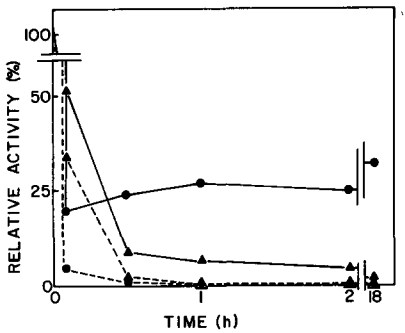


Fig. 1. Inactivation of pyruvate carboxylase by PHMB and by DTNB. Pyruvate carboxylase (specific activity 10.9) was incubated with PHMB (●) and DTNB (▲). The molar ratio of inhibitor to enzyme was 12 (—, 0.3 mg of enzyme/ml) or 36 (— — —, 0.1 mg of enzyme/ml). In all cases, the final inhibitor concentration was 5 μM. Enzymatic activities were determined on aliquots of the incubation mixtures at the indicated times of incubation. Activities are expressed as percentages of the activity at the different incubation times of a control without inhibitor. The activity of the control decreased about 25% in 18 h.

a wedge window and the control enzyme in the standard cell to facilitate direct comparison. Areas of peaks were drawn on paper using a photographic enlarger, measured with a planimeter and corrected for radial dilution.

RESULTS

Inactivation of pyruvate carboxylase by sulfhydryl-group reagents

The SH-group reagents PHMB and DTNB are powerful inhibitors of pyruvate carboxylase (Fig. 1). After a 5-min incubation, at a molar ratio of inhibitor to enzyme (tetramer) equal to 12, PHMB caused 80% inactivation, while DTNB produced only 48%. Under these conditions, however, the extent of the PHMB inactivation remained nearly constant with time, while that of DTNB increased to about 95% after 2 h of incubation. When the molar ratio of PHMB or DTNB to enzyme was

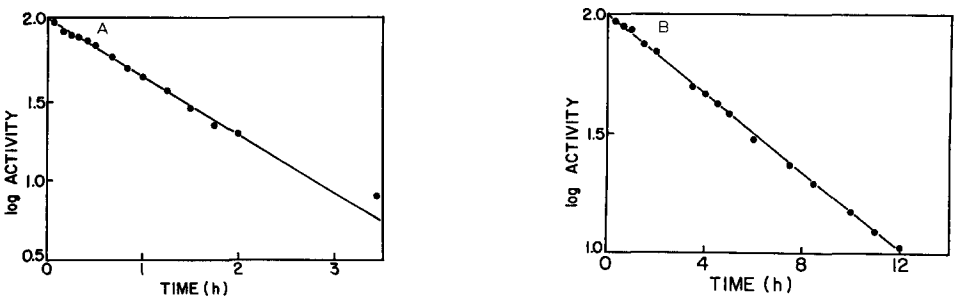


Fig. 2. Inactivation of pyruvate carboxylase by iodoacetate (A) and by cystine (B). Enzymatic activities were determined on aliquots of the incubation mixtures at the indicated times of incubation. Activities are expressed as percentages of the activity of the corresponding control without inhibitor. (A). Pyruvate carboxylase (specific activity 26.8; 0.25 mg/ml) was incubated with iodoacetate (10 mM). (B). Pyruvate carboxylase (specific activity 27.0; 0.25 mg/ml) was incubated with cystine (0.83 mM).

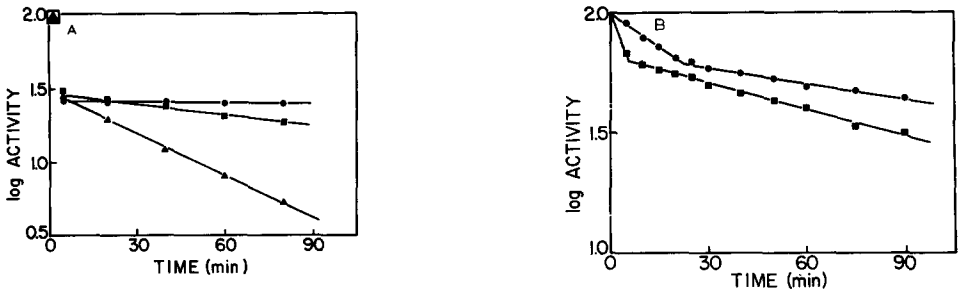


Fig. 3. Inactivation of pyruvate carboxylase by *N*-ethylmaleimide (A) and by iodoacetamide (B). Enzymatic activities were determined on aliquots of the incubation mixtures at the indicated times of incubation. Activities are expressed as percentages of the activity of the corresponding control without inhibitor. (A). Pyruvate carboxylase (specific activity 11.3; 0.27 mg/ml) was incubated with *N*-ethylmaleimide (●—●, 0.4 mM; ■—■, 2 mM; ▲—▲, 10 mM). (B). Pyruvate carboxylase (specific activity 26.6; 0.25 mg/ml) was incubated with iodoacetamide (●—●, 5 mM; ■—■, 20 mM).

increased to 36, by decreasing the enzyme concentration, complete inactivation occurred in both cases within 1 h.

Iodoacetamide, iodoacetate, cystine and *N*-ethylmaleimide are effective inhibitors when their concentration is much higher than that of PHMB and DTNB in Fig. 1. The inactivation produced by iodoacetate and cystine followed pseudo-first-order kinetics with time to less than 10% activity (Fig. 2). In contrast, inactivation by *N*-ethylmaleimide and iodoacetamide proceeded in two steps (Fig. 3). The first phase accounted for about 75% inactivation in the case of *N*-ethylmaleimide and about 35% in that of iodoacetamide, regardless of inhibitor concentration, while the second step produced complete inactivation in both cases. The first step of inactivation seems to correspond to the formation of a partially inactivated enzyme, which is completely inactivated by a further binding of reagent. Thus, it appears that there are two (or more) kinds of sulfhydryl groups on pyruvate carboxylase which differ in their rate of reaction with *N*-ethylmaleimide or iodoacetamide and in their effect upon enzymatic activity. If pyruvate carboxylase possessed different kinds of subunits, the possibility exists of a correlation of this pattern of inactivation with reaction of *N*-ethylmaleimide or iodoacetamide on one subunit before another.

Since the rate of inactivation during the second phase depends upon the concentration of inhibitor, additional sulfhydryl groups must react to cause complete inactivation. The concentration of *N*-ethylmaleimide could be adjusted so that practically no further inactivation occurred after the first 75%, at least in 2 h. This excludes a conformational change after the first phase of inactivation as the only cause of the second phase of inactivation.

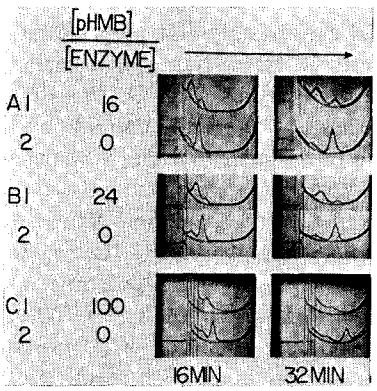
Effect of sulfhydryl reagents on the sedimentation properties of pyruvate carboxylase

The inactivation of pyruvate carboxylase by PHMB, under certain conditions, is accompanied by a dissociation of the tetramers to monomers. The changes in the ultracentrifugal pattern of pyruvate carboxylase, upon incubation with different amounts of PHMB, are shown in Fig. 4. When the molar ratio of PHMB to enzyme was equal to 16 (Fig. 4A), there was a change of material from the fast-moving (15 S) to the slow-moving component (7 S). The activity of the enzyme dropped to 25% of

that of a control without PHMB. As the molar ratio was increased from 16 (Fig. 4A) to 24 (Fig. 4B) and 100 (Fig. 4C), there was a progressive decrease in the amount of the fast-moving component (15 S) and a formation of the slow-moving one (7 S). At each PHMB level there was a concomitant increase in the extent of the inhibition. At the highest level of PHMB (Fig. 4C), there was also a considerable formation of aggregates (not shown).

In the experiments of Fig. 4, the centrifuge reached full speed about 30 min after the addition of PHMB to the enzyme. When the enzyme was incubated for 3 h before centrifugation at the lowest molar ratio (16) of inhibitor to enzyme, no further change took place in the sedimentation pattern or in the extent of inactivation. On the contrary, at a molar ratio of 100 and incubation for 5 h prior to centrifugation, both the 15-S and the 7-S peaks disappeared and heterogeneous aggregates were observed during the run. The most conspicuous aggregate had a sedimentation coefficient of about 40 S. Whether the formation of 7-S^{20}_{w} monomers is required before aggregation can occur, as it would appear, is uncertain.

DTNB also caused dissociation of pyruvate carboxylase to its monomers. The incubation of the enzyme with DTNB (at a molar ratio of DTNB to enzyme equal to 12) produced a decrease of the 15-S peak and an increase of 7-S material (Fig. 5A).



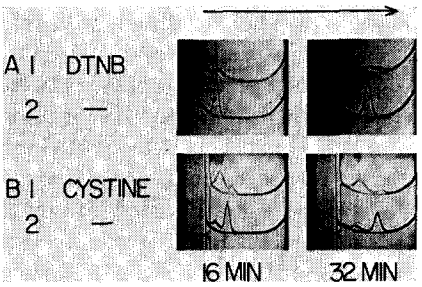


Fig. 4. Effects of different molar ratios of PHMB to enzyme on the sedimentation pattern of pyruvate carboxylase. The preparations numbered 1 were those incubated with PHMB, and those numbered 2 were controls without PHMB. Pyruvate carboxylase (specific activity 18.0 in A, 14.1 in B, and 15.4 in C; 1.3 mg/ml) was incubated with different amounts of PHMB (molar ratio of PHMB to enzyme equal to 16 in A1, 24 in B1, and 100 in C1) with different degrees of inactivation (75% in A1, 87% in B1, and 100% in C1). The preparations reached full speed in the ultracentrifuge soon after the addition of PHMB (32 min for A, 47 min for B, and 28 min for C). Pictures were taken at the following bar angles: 55° (16 min) and 50° (32 min) for A, 60° for B, and 65° for C. The direction of sedimentation and the times after reaching full speed (52 000 rev./min for A, and 50 740 for B and C) are shown in the figure.

Fig. 5. Effect of DTNB and cystine on the sedimentation pattern of pyruvate carboxylase. (A). Pyruvate carboxylase (specific activity 26.8; 1.1 mg/ml) was incubated with DTNB (molar ratio of DTNB to enzyme equal to 12) with 72% inactivation. This preparation (A1) and the control without DTNB (A2) reached full speed in the ultracentrifuge 32 min after the addition of DTNB. (B). Pyruvate carboxylase (specific activity 14.1; 1.3 mg/ml) was incubated with cystine at a concentration 2.1 mM (molar ratio of cystine to enzyme equal to 1060) with 79% inactivation. This preparation (B1) and the control without cystine (B2) reached full speed in the ultracentrifuge 3.5 h after the addition of cystine. Pictures were taken at the following bar angles: 65° (16 min) and 60° (32 min) for A, and 60° for B. The direction of sedimentation and the times after reaching full speed (52 000 rev./min for A and 50 740 for B) are shown in the figure.

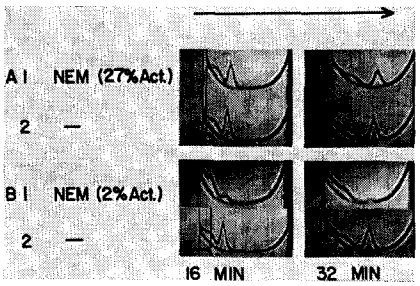


Fig. 6. Effect of *N*-ethylmaleimide (NEM) on the sedimentation pattern of pyruvate carboxylase. Pyruvate carboxylase (A: specific activity 11.2, 1.2 mg/ml; B: specific activity 13.7, 1.15 mg/ml) was incubated with *N*-ethylmaleimide at two different concentrations (0.4 mM in A1, and 10 mM in B1). The preparations reached full speed in the ultracentrifuge about 3 h after the corresponding addition of *N*-ethylmaleimide. At this time, the activity of preparation A1 was 27%, and that of B1 2% of the activities of the corresponding controls (preparations A2 and B2). Pictures were taken at the following bar angles: 55° for A, 50° for B1, and 60° (16 min) and 55° (32 min) for B2. The direction of the sedimentation and the times after reaching full speed (50 740 rev./min) are shown in the figure.

When the molar ratio of DTNB to enzyme was increased to 200, both the 15-S and the 7-S peaks disappeared and rapidly moving aggregates were observed during centrifugation. Incubation of pyruvate carboxylase with cystine produced a conversion of tetramers to monomers proportional to the extent of inactivation (Fig. 5B). No aggregation was apparent.

When studying the effect of *N*-ethylmaleimide on the sedimentation pattern of the enzyme, its two-step inactivation pattern (Fig. 3A) was taken into account. During the first step of inactivation, no significant change in the sedimentation pattern took place (Fig. 6A), indicating that the partially inactivated enzyme, like the native one, has a tetrameric structure. The second step of inactivation was accompanied by a formation of fast-moving aggregates (Fig. 6B). No increase was observed in the amount of monomers, with respect to the control without *N*-ethylmaleimide, indicating that either the aggregates were formed by direct aggregation of the tetramers or that the intermediate monomers were too unstable to be detected.

Interrelation of the effects of different sulfhydryl-group reagents on pyruvate carboxylase

The partial inactivation of pyruvate carboxylase by iodoacetamide protects the enzyme against the greater inactivation produced by DTNB or *N*-ethylmaleimide. The enzyme was preincubated with iodoacetamide prior to the addition of *N*-ethylmaleimide or DTNB. After the addition of these last reagents, the samples preincubated with iodoacetamide showed higher enzymatic activity than the controls without iodoacetamide (Table I). The results show that the effects of SH-group reagents on the enzymatic activity are not independent of each other. The resistance toward further inactivation by *N*-ethylmaleimide or DTNB of the enzyme partially inactivated by iodoacetamide is consistent with the reaction of the latter reagent with the same sulfhydryl groups that could react with *N*-ethylmaleimide or DTNB and cause inactivation. Other interpretations are also possible.

In a similar experiment protection against changes in quaternary structure was observed. The partial inactivation of the enzyme by *N*-ethylmaleimide prevented the

TABLE I

PROTECTION BY IODOACETAMIDE AGAINST INACTIVATION BY DTNB OR *N*-ETHYLMALEIMIDE

Pyruvate carboxylase (specific activity 13.6, 0.25 mg/ml) was preincubated with iodoacetamide for 12 min, prior to the addition of *N*-ethylmaleimide or DTNB. The activities were determined 30 min (A) and 20 min (B) after the addition of iodoacetamide to the enzyme.

Additions	*Relative activity (%)*
A. 1. None	100
2. 15 mM iodoacetamide	57
3. 0.4 mM *N*-ethylmaleimide	22
4. 15 mM iodoacetamide + 0.4 mM *N*-ethylmaleimide	55
B. 1. None	100
2. 9 mM iodoacetamide	64
3. 6 μM DTNB	20
4. 9 mM iodoacetamide + 6 μM DTNB	64

changes in the sedimentation pattern brought about by DTNB (Fig. 7). This stabilization of the quaternary structure of the enzyme shows the interrelation of the effects of different sulfhydryl-group reagents.

Reversibility of the effects of PHMB, cystine and DTNB on pyruvate carboxylase

The addition of different thiols to a preparation of pyruvate carboxylase inactivated by PHMB was followed by an almost complete reactivation of the enzyme (Table II). The maximal reactivation was nearly attained after one hour of incubation with thiol. The thiol most effective for reactivation was DTE.

When cysteine was added to the PHMB-inactivated enzyme, after an initial reactivation, the activity decreased considerably (Table II). Because of this apparent anomalous result, the effects of cysteine and cystine on an uninhibited enzyme preparation were studied. The addition of cysteine or cystine to a preparation of pyruvate carboxylase caused an inactivation that increased with time and was partially reversed by addition of DTE (Fig. 8). The extent of the reactivation by DTE decreased with

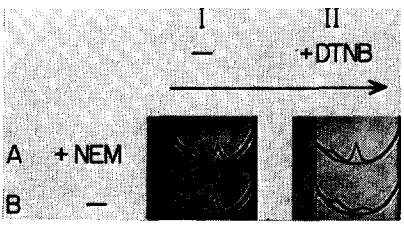


Fig. 7. Protection by *N*-ethylmaleimide against the change in the sedimentation pattern produced by DTNB. Pyruvate carboxylase (specific activity 11.2; 1.2 mg/ml) was incubated with *N*-ethylmaleimide (NEM) (10 mM) for 12 min (preparations AI and AII), with a loss of activity of 78%. At this time, DTNB (molar ratio of DTNB to enzyme equal to 7) was added to preparation AII and to a control without *N*-ethylmaleimide (BII). The preparations reached full speed in the ultracentrifuge 1 h (AII and BII) and 3 h (AI and BI) after the addition of *N*-ethylmaleimide. Pictures were taken at a bar angle of 60°, 32 min after reaching full speed (50 740 rev./min). The direction of the sedimentation is shown in the figure.

TABLE II

REACTIVATION BY THIOLS OF THE PHMB-INACTIVATED PYRUVATE CARBOXYLASE

Pyruvate carboxylase (specific activity 10.5, 0.23 mg/ml) was preincubated with PHMB (molar ratio of PHMB to enzyme equal to 100) for 5 min with consequent inactivation, prior to the addition of the thiols. After incubation with a given thiol for the indicated time, the enzymatic activity was determined. Activities are expressed as percentages of the activity of a control with neither PHMB nor thiol.

Addition (2 mM)	*Time of incubation with the thiol:*	*Relative activity (%)*		
		5 min	*1 h*	*18 h*
None		0	0	0
Dithioerythritol		76	92	97
2-Mercaptoethanol		74	85	91
Thioglycollic acid		76	87	81
Glutathione		74	88	79
Cysteine		77	82	8

time in both cases. It seems likely that cysteine is oxidized to cystine in the incubation mixture, and that the latter compound is the one responsible for the observed inactivation.

The proportion of reversible and irreversible inactivation by thiol reagents was studied by measuring the activity after incubating the inhibited enzyme with DTE. Irreversible inactivation is defined as the inactivation that remains after incubation with 2 mM DTE for 1 h. The irreversible inactivation caused by PHMB increased with the time of incubation prior to the addition of DTE and with the molar ratio of inhibitor to enzyme (Fig. 9). The irreversible inactivation produced by DTNB (Fig. 9) proceeded more slowly than that caused by PHMB under similar conditions. Thus, the addition to the enzyme of PHMB, DTNB or cystine (Fig. 8, right) produces both a DTE-reversible and a DTE-irreversible inhibition.

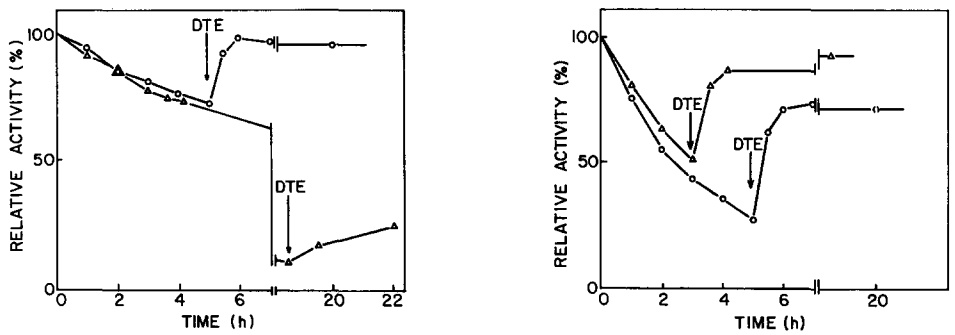


Fig. 8. Inactivation of pyruvate carboxylase by addition of cysteine (left), and cystine (right), and reactivation by DTE. Pyruvate carboxylase (specific activity 9.7, △—△, and 16.1, ○—○; 0.25 mg/ml) was incubated with 1.7 mM cysteine (left), and with 0.85 mM cystine (right). At the times indicated by the arrows, DTE (17 mM) was added to the incubation mixtures. Enzymatic activities were determined on aliquots of the incubation mixtures at the indicated times of incubation. They are expressed as percentages of the activity of a control without cysteine or cystine.

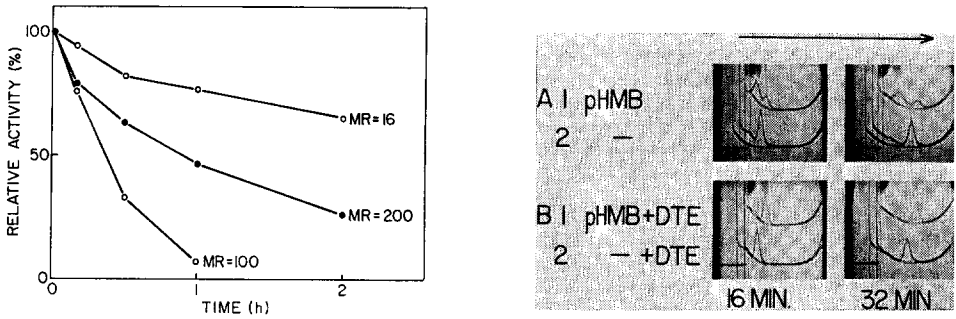


Fig. 9. Irreversible inactivation of pyruvate carboxylase by PHMB and DTNB. Pyruvate carboxylase (specific activity 17, 15.9 and 16.5; 0.23 mg/ml) was incubated with PHMB, and with DTNB, for the times indicated in the figure after which DTE (2 mM) was added. After incubation with DTE for 1 h, aliquots were withdrawn for determination of enzymatic activity. Activities are expressed as percentages of the activity of a control without inhibitor. MR indicates molar ratio of inhibitor to enzyme. ○—○, PHMB; ●—●, DTNB.

Fig. 10. Effect of reactivation by DTE of PHMB-inactivated pyruvate carboxylase on the sedimentation pattern. (A) Pyruvate carboxylase (specific activity 21.6; 1.3 mg/ml) was incubated with PHMB (molar ratio of PHMB to enzyme equal to 20) with 74% inactivation. This preparation (A1), and a control without PHMB (A2) reached full speed in the ultracentrifuge 40 min after the addition of PHMB. (B) 1 h after the addition of PHMB to the enzyme, DTE (0.9 mM) was added to preparations identical to A1 and A2. 2.5 h after the addition of DTE, these preparations reached full speed in the ultracentrifuge. The upper schlieren pattern corresponds to the preparation that had been incubated with PHMB (B1), and the lower one to the control without PHMB (B2). Preparation B1 had 95% of the activity of the control (B2). Pictures were taken at a bar angle equal to 60°. The direction of sedimentation and the times, after reaching full speed (50 740 rev./min) are shown in the figure.

The reactivation of the PHMB-inactivated pyruvate carboxylase by DTE is accompanied by a recovery of the original sedimentation pattern. The enzyme was incubated with PHMB, molar ratio of PHMB to enzyme equal to 20, with partial inactivation and a decrease in the amount of the 15-S component (Fig. 10A). Addition of DTE (0.9 mM) to the PHMB-inactivated preparation caused reactivation and recovery of the original sedimentation pattern (Fig. 10B).

DISCUSSION

The investigation of the sulfhydryl groups of an enzyme with a large number of such groups leads to several difficulties. Since there appears to be a range of chemical reactivities which change with different reagents, it becomes difficult to define which sulfhydryls or even how many of them influence a certain property. The investigation of the chemical reactivity of other functional groups is also complicated, since sulfhydryl groups will react with many different reagents. To further complicate matters, it is very likely that indirect, conformational effects dominate any possible direct effect of sulfhydryl-group reagents on such a large unstable enzyme as pyruvate carboxylase. However, the study of the structural changes that accompany inactivation, and the dependence of these changes on the nature of the reagent used, might shed some light on the way in which the structure depends on the particular chemical moieties of the enzyme.

PHMB promotes dissociation of pyruvate carboxylase; under the conditions

used, about 50% dissociation occurs when 16 moles of PHMB have been added per mole of pyruvate carboxylase. The monomers of pyruvate carboxylase resulting from the reaction of PHMB with the tetramer can form high-molecular-weight aggregates. However, further addition of inhibitor is necessary to bring about significant aggregation, since there was no additional formation of aggregates with a molar ratio of PHMB to enzyme equal to 16 in 3 h. With a high PHMB concentration, molar ratio of PHMB to enzyme equal to 100, aggregates appear early during incubation. DTNB produces similar effects. However, the formation of disulfide bridges between sulfhydryls of the enzyme[11] introduces complications at low molar ratios of DTNB to enzyme.

In a subsequent publication[11] it will be shown that at the end of the first phase of inactivation by *N*-ethylmaleimide, about 40 of the 55 sulfhydryls of the enzyme have reacted, as determined by spectrophotometric titration with *N*-ethylmaleimide. That this occurs without a complete loss of activity or a significant change in the quaternary structure indicates that most of the sulfhydryls of pyruvate carboxylase are on the surface of the enzyme and do not play an essential role in the maintenance of the structure of the active site. The protection by *N*-ethylmaleimide against the change in the quaternary structure effected by DTNB is reasonable, because most of the sulfhydryls of the enzyme are already bound to *N*-ethylmaleimide and the rest might be inaccessible to DTNB or react very slowly.

There is, then, a striking difference in the effects of different sulfhydryl reagents on the quaternary structure of the enzyme. Reaction of about 40 sulfhydryls of the enzyme with *N*-ethylmaleimide produces partially active tetramers, while the reaction

TABLE III

SUMMARY OF THE EFFECTS OF DIFFERENT SULFHYDRYL REAGENTS ON THE QUATERNARY STRUCTURE

Monomer and tetramer concentrations were calculated from area measurements of the ultracentrifugal schlieren patterns. In all cases, the protein concentration was between 1.0 and 1.3 mg/ml. Column 3, the relative amount of tetramers, was obtained by dividing the amount of tetramers in the inactivated preparation by the amount of tetramers in the control. Column 4, ratio of Column 3 to Column 2. Column 5, ratio of the increase in monomers to the decrease in tetramers. The increase in monomers is equal to the amount of monomers in the inactivated preparation *minus* the amount of monomers in the control. The decrease in tetramers is equal to the amount of tetramers in the control *minus* the amount of tetramers in the treated preparation.

Reagent	*(1) Specific activity of the untreated preparation*	*(2) Relative activity of the treated preparation*	*(3) Relative amount of tetramers*	*(4) Relative amount of tetramers / relative activity*	*(5) Increase in monomers / decrease in tetramers*
Cystine (2.1 mM)	14.1	0.21	0.21	1.0	1.0
PHMB (MR* = 16)	18.0	0.27	0.40	1.5	0.9
DTNB (MR* = 12)	26.8	0.28	0.52	1.9	0.9
Iodoacetate (10 mM)	14.0	0.16	0.61	3.8	0.5
Iodoacetamide (10 mM)	13.7	0.39	0.53	1.4	0.3
4,4′-Dithiodipyridine (MR* = 10)	14.0	0.15	0.57	3.8	0.2
N-ethylmaleimide (10 mM)	13.7	0.02	0.18	9.0	0.04

* MR, molar ratio of inhibitor to enzyme.

of approximately the same number of sulfhydryl groups with PHMB is accompanied by formation of inactive monomers.

The data illustrating these points are summarized in Table III. Column 4 relates the amount of tetramer to the activity in the inhibited preparation and would remain at a value of 1.0 if the inactivation caused by the sulfhydryl reagents were strictly due to the breakdown of the tetrameric structure of the enzyme. However, the experimental values obtained were greater than one, although in the case of cystine the value was close to 1.0. These values higher than one indicate the existence of partially or completely inactive tetramers originated by the binding of the reagent to the enzyme. That is, the activity decreased to a greater extent than did the tetramer fraction. However, a complication arises from the presence of inactive tetramers in the native preparation[3]. If we take into account this fact and the estimation of a maximal activity of the enzyme of about 35 units per mg (ref. 1), the values of Column 4 greater than 1.0 could be obtained without the formation of inactive tetramers from active ones. This could occur if the inactive tetramers present in the native preparation disappeared, by effect of the reagent, at a lower rate than the active ones. However, during the first phase of inactivation by *N*-ethylmaleimide (not shown in Table III), the formation of partially inactivated tetramers is quite clear (Fig. 6A).

Column 5 of Table III shows what has happened to the tetramers that have disappeared. If the decrease in tetramers is exactly equal to the amount of monomers formed the ratio will be 1.0, but if aggregation has occurred, the ratio will be less than one, *i.e.* the amount of monomers formed is less than the amount of tetramers that has disappeared. There is a certain degree of correlation between the nature of the reagent used and the effect on this ratio (Column 5). Reagents that attach a charged group to the enzyme (PHMB, DTNB, cystine and iodoacetate) give a value closer to one for the index of Column 5 than those that introduce an uncharged group (*N*-ethylmaleimide, iodoacetamide, and 4,4'-dithiodipyridine). Therefore, it can be said that the charged-group reagents tend to produce more stable monomers than the uncharged-group reagents, or that the change in the sedimentation pattern caused by uncharged-group reagents is mainly due to a direct aggregation of tetramers without a monomeric intermediate.

It is interesting that four different factors—low temperature[1–3], low concentration of urea[3], certain sulfhydryl reagents and possibly a pH equal to 8 (ref. 3)—promote dissociation of chicken pyruvate carboxylase, suggesting that the forces that maintain the tetrameric structure are critically balanced. The cold dissociation[1–3] seems to indicate that the contact regions among the four subunits of the enzyme have a partially hydrophobic nature[12,13]. The attachment of charged groups to sulfhydryls in the contact regions could effect a weakening of the forces that keep the subunits together and consequently a separation of subunits. This model would explain the differences in the inactivation and the change of the quaternary structure brought about by the different reagents used, and the protection obtained with *N*-ethylmaleimide or iodoacetamide.

The reversibility of the inactivation caused by some reagents suggests a structural stability of the inactivated enzyme. No gross changes have probably occurred in the fraction of the enzyme molecules that is capable of being reactivated; the slow increase of the irreversibly inactivated enzyme may indicate that other, more drastic, changes in the secondary and tertiary structures are occurring. No correlation was

found between the amount of irreversibly inactivated enzyme and the amount of aggregated material observed with the ultracentrifuge.

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