Substrate- and Inhibitor-induced Conformational Changes in Enzymes Measured by Tritium-Hydrogen Exchange

II. YEAST PYRUVATE DECARBOXYLASE*

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SUMMARY

The effects of substrates and inhibitors on the conformation of yeast cytoplasmic pyruvate decarboxylase (2-oxo-acid decarboxylase, EC 4.1.1.1) has been examined using the technique of automated tritium-hydrogen exchange. This experimental approach, as used in this study, reflects primarily the number of solvent-inaccessible or "core" hydrogens in the protein. Yeast cytoplasmic pyruvate decarboxylase is a multimeric enzyme, molecular weight 208,000, and contains thiamine pyrophosphate as a co-factor at the active site. We find that 850 out of a total of 1600 amide hydrogens are measurable by tritium exchange at pH 6.5, 0°. There are 225 (or 14% of the total) very slowly exchanging hydrogens which we believe arise either from solvent-inaccessible regions of the subunits or from interface areas between the subunits.

Upon addition of substrate, pyruvate, inhibitor, α-ketoglutarate, or mercuric chloride, a marked conformational change occurs in which 50% of the very slowly exchanging hydrogens become rapidly exchanging. Lactate and formaldehyde have no effect. Mercuric chloride causes a partial conformational change, but the enzyme is still capable of binding pyruvate and exhibiting a further release of slowly exchanging hydrogens. The effects of pyruvate and mercuric chloride are not additive; therefore, we believe that they are acting at common areas on the enzyme.

The enzyme is not dissociated into subunits by the substrate. Thus, this enzyme has a conformationally regulated accessibility to its active site. It is proposed that the conformational changes we observe may be very similar to those obtained during allosteric modification of enzymes and may involve a reorganization of subunit interfaces.

The importance of understanding the molecular processes occurring in enzymes upon interaction with substrates or inhibitors is widely accepted, and several conformational probes have been utilized to this end. Tritium-hydrogen exchange is an experimental approach with much potential for following conformational changes in enzymes. This technique measures the rate with which amide protons exchange with hydrogen in the solvent (1). In the case of proteins the predominant exchange site is the backbone peptide N-H proton. A primary determinant for exchange is the formation of a hydrogen bond between the peptide N-H and water. A marked slowing of the exchange rate may therefore result from either participation of the peptide hydrogen in stable intra- or intermolecular hydrogen bonding or from the location of the potentially exchangeable group in a solvent-inaccessible region, or both. Thus, if substrate or inhibitor alters the conformation of an enzyme such that there is increased or decreased accessibility of amide hydrogens to the solvent, then a change in the rate of exchange or in the number of very slowly exchanging hydrogens may be observed. The development of an automated sample handling-tritium-hydrogen exchange system provides a method to readily probe questions related to conformational changes in enzymes occurring upon binding of substrates or inhibitors. We have used automated tritium-hydrogen exchange to measure conformational changes occurring in pyruvate decarboxylase. This cytoplasmic enzyme, isolated from yeast, has been recently purified and shown to undergo reversible pH dependent dissociation from a molecular weight of 208,000 (under optimum enzymatic conditions) to 36% the molecular weight at pH 8 (2). Thiamine pyrophosphatase has been shown to be a required co-factor (3–5). Dissociation of the enzyme at pH 8.0, removal of the TPP by Sephadex G-25 chromatography, and reassociation in the absence of the TPP leads to a partially aggregated preparation with no detectable enzymatic activity (2). Thus, in addition to a catalytic function, TPP has an additional distinct role in the reassociation of the subunits.

The interaction of TPP with enzyme has exhibited some rather unusual properties, e.g. (a) TPP does not dissociate from the holoenzyme at pH 6 to 7 (the enzymatically active range) (6, 7) and (b) the presence of a large excess of TPP is required to recover total activity upon reconstitution of the holoenzyme (6, 7).

* The abbreviation used is: TPP, thiamine pyrophosphate.
8). These observations are consistent with a hypothesis that TPP is located in the interior of the active dimer protein. Since the initial step in the enzymatic mechanism is the formation of the TPP-pyruvate adduct (9–13), it is reasonable to suggest that a reorganization of the protein might occur when the substrate is added.

Consistent with this expectation we have found from automated tritium-hydrogen exchange studies a marked conformational change occurring in this enzyme upon addition of either substrate (pyruvate) or inhibitor (α-ketoglutarate). We have also obtained evidence that one or more sulfhydryl groups are located in the area of the active site. Reaction of the enzyme with mercury caused a partial unfolding of the enzyme molecule; subsequent addition of pyruvate then appeared to complete the unfolding as evidenced by a further increased exchange of very slowly exchanging hydrogens. Mercury is a known inhibitor of pyruvate dehydrogenase (5). The enzyme is specific for α-keto acids and in agreement with this we find known inhibitor of pyruvate dehydrogenase (5). The enzyme exchange of very slowly exchanging hydrogens. Pyruvate, as well as α-ketoglutarate, also absorbs strongly at 225 nm, the wave length used to monitor enzyme concentration. Pyruvate, as well as α-ketoglutarate, absorbs very strongly at 225 nm, the wave length used to monitor enzyme concentration. The control decarboxylase enzyme, as the ammonium sulfate precipitate, was dissolved in and dialyzed overnight against 0.02 M potassium phosphate buffer, pH 6.5, containing 0.05 M MgCl₂, 0.05 M TTP, and 5 mM mercaptoethanol. The enzyme was eluted with 0.02 M potassium phosphate, 0.1 M KCl, pH 6.5 buffer. For the substrate test, a sample, treated as above, was incubated for 30 min at 5°C with a buffer containing 50 mM pyruvate in addition to the phosphate, magnesium chloride, TTP, and mercaptoethanol as listed above. The column was eluted with 0.02 M potassium phosphate, 0.1 M KCl, pH 6.5 buffer. For the substrate test, a sample, treated as above, was incubated for 30 min at 5°C with a buffer containing 50 mM pyruvate in addition to the phosphate, magnesium chloride, TTP, and mercaptoethanol as listed above. The column was equilibrated with 0.02 M potassium phosphate, 0.1 M KCl, pH 6.5 buffer, and the enzyme was eluted with the same buffer. The presence of the pyruvate did not alter the column characteristics. Protein position was determined by optical density at 280 nm measurements and, for the pyruvate experiment, also by Lowry determinations of protein concentration in and around the apparent peak position.

**Results**

**Enzyme Control and Substrate**—The exchange curve for the enzyme alone is given in Fig. 1. These results contain data from multiple experiments, thus demonstrating the high reproducibility of the automated system. The terminal part of the curve may be extrapolated to the zero time ordinate and gives the total number of very slowly exchanging hydrogens, namely 225. Addition of succinate did not affect the exchange curves. Upon addition of pyruvate, Fig. 2, there is a marked increase in the exchange rate of approximately 100 very slowly exchanging hydrogens. Pyruvate, as well as α-ketoglutarate, absorbs very strongly at 225 nm, the wave length used to monitor enzyme concentration. The second column separation should separate all of the free, low molecular weight substrate from the enzyme; however, we observed a marked skewing of the pyruvate peak into the enzyme-protein peak. This was interpreted as reflecting association of the substrate with the enzyme since lactate, also highly absorbing at 225 nm, did not skew into the enzyme peak nor did it effect the exchange kinetics of the enzyme. The skewing of the pyruvate peak diminished greatly as substrate was utilized; however, even after 1600 min there was a slightly altered elution absorbance profile of the enzyme. This altered absorbance profile complicated the determination of the enzyme concentration and resulted in the scatter shown in Fig. 2. We were able to handle this complication in the following two ways. First, in these experiments we used only the
The sequence of addition of various perturbants. The experiment was always begun by passing 0.2 ml of tritiated enzyme solution through the first column which removed all of the free, unbound tritium. The enzyme peak was distributed over three fractions. Small aliquots of each fraction were diluted with buffer and analyzed for protein and tritium concentration. These points were averaged to get the first data point on the exchange curve (P). The bulk of the material (0.6 to 0.75 ml) from the three fractions was pooled and used for the remaining exchange points. This pool contained approximately 1.3 mg of enzyme (6.22 pmoles) in a volume of 0.6 to 0.75 ml. For time points, 40 µl of the pool was automatically passed through a second column. The sequence of addition of the perturbants in each experiment is indicated by the arrow. The actual time of addition is indicated by the times listed under each flow line. The concentrations of each perturbant used is as follows: 30 µmoles of sodium succinate, pH 6.5 (Suc); 40 µmoles of potassium pyruvate, pH 6.5 (Pyr); 40 µmoles of potassium α-ketoglutarate, pH 6.5 (α-ket); 40 µmoles of sodium lactate, pH 6.5 (Lac); 15 nmoles of mercuric chloride (HgCl₂), and 37 pmoles of formaldehyde, pH 6.5, adjusted (Form).

Table I

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<tr>
<th>Exchange points</th>
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Fig. 1. Automated tritium-hydrogen exchange curves for pyruvate decarboxylase. The experiment was run as described in “Experimental Procedure” and in the legend to Table I. Succinate, lactate, and formaldehyde were added at times indicated in Table I. The ordinate is H₂/mmol, hydrogens remaining unexchanged per mole of 208,000 molecular weight enzyme.

that there is substrate-facilitated exchange of at least 100 previously very slowly exchanging hydrogens. There also appears to be an increased exchange rate of the remaining slow hydrogens.

Inhibitor Substrate Controls—Lactate is the reduced form of pyruvate and was used both to test for specificity of binding of α-keto acids by the enzyme and to eliminate any artifacts resulting from the high ultraviolet absorbance of the substrate and inhibitor. Lactate has no effect on the exchange kinetics and gave no indication of binding to the enzyme, as described above (Fig. 1). α-Ketoglutarate has been shown to be an inhibitor of pyruvate decarboxylase (16) and is not decarboxylated by the enzyme. Addition of α-ketoglutarate to the enzyme showed a marked effect on the observed exchange curves (Fig. 2). In addition, we observed a pronounced skewing of the inhibitor peak into the enzyme peak, as found for pyruvate, indicating that inhibitor interacts with the enzyme. The magnitude of this skewing did not diminish throughout the run as with pyruvate, which is consistent with the observation that the inhibitor is not decarboxylated. The observed exchange kinetics of the enzyme with inhibitor added is remarkably similar to that with substrate. The possibility existed that the effect we were observing was a result of general Schiff-base formation of the car-

3 A. D. Gounaris, unpublished results.
Fig. 2. Automated tritium hydrogen exchange curves for pyruvate decarboxylase. The experimental details of the addition of pyruvate, succinate, α-ketoglutarate, and mercuric chloride are given in Table I. — in the figure is taken from the curve in Fig. 1 and represents the native exchange curve. A, the bony moiety of the substrate with the primary amino groups of lysine on the protein, and not necessarily an interaction occurring at the active site. To exclude this possibility we added a high concentration of formaldehyde to an exchanging solution in an experimental sequence identical with that used for addition of substrate. As shown in Fig. 1, there is no effect of the addition of formaldehyde on the exchange kinetics of the enzyme. Formaldehyde has been shown to slowly inactivate the enzyme (17). The time scale of this inactivation suggests a slow, catalytically independent reaction. Thus, we may exclude a general Schiff-base reaction as being responsible for the exchange results involving a carbonyl group.

**Mercury-treated Enzyme**—In order to test whether pyruvate would induce a conformational change in an inactive enzyme we utilized the knowledge that there is a sulfhydryl group essential for enzymatic activity (18). Mercuric chloride, a potent inhibitor of enzymatic activity, inactivates the enzyme, presumably by forming the mercaptide of the sulfhydryl. Upon addition of mercuric chloride, Fig. 2, there was a significant drop in the number of very slowly exchanging hydrogens. The magnitude of the effect was not as pronounced as with pyruvate or α-ketoglutarate, and appeared to be plateauing at a level intermediate between that of the control curve and the substrate and inhibitor curves. The addition of pyruvate, 60 min after mercury, see Table I and Fig. 2, caused a further drop in the very slowly exchanging hydrogens to the level found with pyruvate alone. The effect of pyruvate and mercuric chloride are not additive since the final exchange curve was very similar to that with pyruvate alone.

**Test of Substrate-induced Dissociation of Enzyme**—One possible explanation of the observed decrease in the number of very slowly exchanging hydrogens with addition of substrate or inhibitor was that these specific ligands shifted a pre-existing equilibrium between a native, dimeric enzyme and active but dissociated subunits. Such a dissociation would result in a greatly increased solvent accessibility of all the surfaces of the subunits previously forming the interfaces between the subunits. Thus, previously slowly exchanging, solvent-inaccessible hydrogens would become rapidly exchanging, solvent-accessible sites. This effect would obscure any conformational changes. Sephadex G-200 chromatography was used to test for simple dissociation of the enzyme. It had already been established (2) that the apparent molecular weight of yeast pyruvate decarboxylase may be determined by Sephadex chromatography with a calibrated column. It was also shown in that study that the elution position of the dissociated subunits coincided with one-half the molecular weight of the native enzyme. As is evident from Fig. 3, the elution position of the enzyme in the presence and absence of substrate is essentially the same. Furthermore, the band width and elution profile of the enzyme is unchanged. This result indicates that simple ligand-induced dissociation of the enzyme does not occur to any significant degree. The presence of the pyruvate in the eluting buffer resulted in a high blank optical density. It is interesting that the enzyme resulted in a diminution of this optical density around the enzyme peak. This, we believe, results from enzymatic decarboxylation of the pyruvate. An additional evidence for enzymatic decarboxylation acetalddehyde was detected using the fuchsin aldehyde reagent in the same tubes where the enzyme was found. This experiment then demonstrates that the enzyme does not undergo ligand-induced dissociation while functionally active.

**DISCUSSION**

**Properties of Enzyme** Enzymatic decarboxylations of pyruvate range from simple decarboxylation producing acetaldehyde and CO₂, catalyzed by cytoplasmic decarboxylase, the enzyme used in this study, to the multicomponent pyruvate dehydrogenase complex associated with aerobic metabolism. The latter pathway, important in mammalian systems, yields acetyl-CoA and CO₂ and acts as a metabolic bridge-spanning glycolysis and the Krebs cycle in glucose catabolism. Pyruvate decarboxylase is but one of many TPP-requiring α-keto decarboxylases which have many enzymatic and structural properties in common. With one known exception (19), that is, the phosphoroclastic reaction, the first critical step that occurs in the breakdown of pyruvate is the decarboxylation to liberate CO₂. This reaction proceeds via addition of pyruvate to the C₂ of the thiazole ring of TPP (9). Decarboxylation leads to the generation of an intermediate referred to as “active aldehyde.” A sulfhydryl group, essential for activity, has been implicated in both the cytoplasmic pyruvate decarboxylase from yeast (18) and the pyruvate decarboxylase component of Escherichia coli.
pyruvate dehydrogenase (20). In addition a comparison of the substrate specificity of our enzyme with pig heart pyruvate dehydrogenase indicates striking similarities, see Table II. Dissociation into $\frac{1}{2}$ the molecular weight at alkaline pH has been reported for several enzymes in this group (2, 20, 22). Such parallel features suggest a related group of enzymes which might well have additional structure-function characteristics in common. Thus, knowledge of substrate- and inhibitor-induced conformational changes observed in the cytoplasmic enzyme might be valuable in defining similar processes in the related enzyme components of multi-enzyme complexes.

**Relevance of Exchange Studies to Enzymes**—Tritium-hydrogen exchange can define the extent of secondary structure in poly-peptides and proteins (1, 23, 24). For a peptide N–H proton to undergo exchange with solvent water, a hydrogen bond must be formed between the water molecule and the amide hydrogen. If the peptide N–H is involved in intra- or intermolecular hydrogen bonding or if it is sterically shielded from the solvent, then the ease with which it may hydrogen bond with the solvent will be reduced proportionately. Thus, a reduced exchange rate would be observed. We have demonstrated that in small peptides a 4- to 10-fold reduction in the exchange rate (relative to nonhydrogen bonded peptide models) is consistent with the presence of secondary structure. In proteins the cooperative conformational changes required for complete solvent accessibility of all peptide hydrogens produces a far greater effect on exchange rates. For example, at pH 6.5, $^{0}^{\circ}$, the pH and temperature of these experiments, a free N–H would be expected to exchange with a $t_{1/2}$ $\approx$ 0.1 min and a solvent-accessible but hydrogen-bonded hydrogen with a $t_{1/2}$ of 0.5 to 1.0 min (15, 25, 26). (These estimates are based on small peptide models.) However, many proteins show exchange rate half-times of hours (23, 24) and we report in this paper a half-time for the slowest hydrogens of 800 min or more. Thus, hydrogen exchange of large proteins reflects the rate of cooperative reversible unfolding to allow availability of the solvent to hydrogen bond with amide hydrogen. With mult-subunit proteins an additional factor may exist; namely, availability of subunit interfaces to the solvent. An interaction such as between substrate, inhibitor, or drug and an enzyme protein, which results in a modification of the latter, may well affect the accessibility of internal peptide hydrogens to the solvent. These effects could either increase or decrease the unperturbed observed exchange rate. The development of an automated tritium-hydrogen exchange system provides a reproducible and reliable method to investigate such alterations in peptide solvent accessibility in enzyme studies. Such information as may be obtainable through automated tritium-hydrogen exchange studies will be valuable both in understanding the mechanisms of action of these enzymes as well as to facilitate the development of more effective inhibitors.

**Summary of Exchange Results with Pyruvate Decarboxylase**—The results of our exchange study of pyruvate decarboxylase can be summarized as follows.

1. In the native enzyme we find that (a) a total of 850 exchangeable hydrogens are observable at pH 6.5, $^{0}^{\circ}$; (b) there are 225 to 230 very slowly exchanging hydrogens with a half-time of exchange equal to 800 min; (c) the exchange curve is nonlinear indicating multiple classes of exchanging hydrogens.

2. Addition of succinate has no effect on exchange rates.

3. Addition of substrate, pyruvate, affects the immediate release of 100 to 110 of the very slowly exchanging hydrogens and increases the exchange rate of the remaining very slowly exchanging hydrogens.

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**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Yeast cytoplasmic pyruvate decarboxylase activity towards α-keto acids</th>
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<tbody>
<tr>
<td>Pyruvate</td>
<td>44% [pig heart pyruvate dehydrogenase (20)] (%)</td>
</tr>
<tr>
<td>α-Ketobutyrate</td>
<td>44% [pig heart pyruvate dehydrogenase (20)] (%)</td>
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<td>α-Ketovalerate</td>
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<td>α-Ketogutarate</td>
<td>44% [pig heart pyruvate dehydrogenase (20)] (%)</td>
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<tr>
<td>Phenylpyruvate</td>
<td>44% [pig heart pyruvate dehydrogenase (20)] (%)</td>
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</table>

* Data from Reference 21.
between the subunits but do not involve actual dissociation of that latter source may make a significant contribution to the new rapidly exchanging hydrogens. First, our observation that almost 50% of the very slowly exchanging hydrogens or exchanging hydrogens. The conformational perturbation of hydrogens released by substrate, inhibitor, or mercury could arise (a) from conformational changes within a subunit, or (b) from changes which result in alterations of the interface surfaces between the subunits but do not involve actual dissociation of the protein, or both. Two major factors lead us to propose that the latter source may make a significant contribution to the new rapidly exchanging hydrogens. First, our observation that almost 50% of the very slowly exchanging hydrogens or core hydrogens was released by ligands argues for a large structural change in the subunits if that were their sole site of origin. Such a change should disrupt some secondary structure of the subunits and therefore should be observable by other conformational probes, such as circular dichroism. In fact, preliminary observations in our laboratories as well as by Ulirich and Wollmer (27) do not disclose any significant change in the circular dichroism pattern. Second, findings from other laboratories have established that the thiamine moiety of thiamine pyrophosphate is bound in a hydrophobic area of the protein molecule (27-29). In addition, as discussed above, it is clearly established that excess thiamine pyrophosphate is needed for reassociation of the isolated subunits to an active enzyme (2, 27). Thus, from studies of pyruvate decarboxylase as well as from model reactions, e.g. the reaction between thiamine and indole derivatives (30, 31), one may conclude that the cofactor is bound to the protein in a solvent-inaccessible region of the molecule. Since the mechanism of the decarboxylation reactions occurring with TPP involves the formation of an adduct between the C3 of the thiazole ring and the C2 carbonyl of the pyruvate, the protein structure must be capable of sufficient flexibility so as to provide access of the substrate to the cofactor.

Protein flexibility may readily be obtained if the cofactor is bound within or very near the subunit interfaces. Thus, a slight realignment of the subunits could, in such a model, be adequate to regulate substrate access to the cofactor. It should be remembered that we do not observe any dissociation of the enzyme in the presence of substrate while the enzyme is functionally active. Thus, simple dissociation of the multimer protein with a concomitant exposure to the solvent of new surfaces cannot be an explanation of the new rapid hydrogens.

Based on these arguments, we believe that upon addition of specific ligands there are conformational changes which result in a slight realignment of subunit interactions. Thus, it is possible that 1 or more moles of substrate (or inhibitor) bind to specific sites on the enzyme and induce conformational changes facilitating sustained enzymatic activity. Our data indicate that such a conformational "gate" (32) requires a-keto acids and does not respond to carboxylic acids, reduced pyruvate, or free aldehyde. The probable involvement of at least one —SH group in this "gate" region is indicated by the effect of mercuric chloride. Formation of the mercaptide might then interfere with the reversible closing of the gate, leaving it partly "open" and increasing the exchange rate of some of the very slowly exchanging hydrogens. Our interpretation of the data argues for a conformationally regulated accessibility to the catalytic site of the enzyme. An analogous interpretation has been recently advanced by Englander and Muel (32) based on their tritium exchange studies of hemoglobin. The process described here may be equivalent to conformational changes associated with allosteric processes, and such a relationship is under active investigation in our laboratory with well defined systems.

Addition of Lignands—The addition of substrate or inhibitor causes the rapid exchange of almost half of the 225 very slowly exchanging hydrogens. The conformational perturbation of this enzyme is essentially equivalent for both substrate and the inhibitor used in this study. This would argue for similar sites of action of both ligands on the enzyme. Since there is an increased exchange rate of a large number of very slowly exchanging hydrogens, both the substrate and inhibitor must promote increased accessibility of the solvent to the "core" region. Thus, there is a conformational reorganization of the enzyme to allow increased solvent penetration. The very slowly exchanging hydrogens released by substrate, inhibitor, or mercury could arise (a) from conformational changes within a subunit, or (b) from changes which result in alterations of the interface surfaces between the subunits but do not involve actual dissociation of the protein, or both. Two major factors lead us to propose that the latter source may make a significant contribution to the new rapidly exchanging hydrogens. First, our observation that almost 50% of the very slowly exchanging hydrogens or core hydrogens was released by ligands argues for a large structural change in the subunits if that were their sole site of origin. Such a change should disrupt some secondary structure of the subunits and therefore should be observable by other conformational probes, such as circular dichroism. In fact, preliminary observations in our laboratories as well as by Ulirich and Wollmer (27) do not disclose any significant change in the circular dichroism pattern. Second, findings from other laboratories have established that the thiamine moiety of thiamine pyrophosphate is bound in a hydrophobic area of the protein molecule (27-29). In addition, as discussed above, it is clearly established that excess thiamine pyrophosphate is needed for reassociation of the isolated subunits to an active enzyme (2, 27). Thus, from studies of pyruvate decarboxylase as well as from model reactions, e.g. the reaction between thiamine and indole derivatives (30, 31), one may conclude that the cofactor is bound to the protein in a solvent-inaccessible region of the molecule. Since the mechanism of the decarboxylation reactions occurring with TPP involves the formation of an adduct between the C3 of the thiazole ring and the C2 carbonyl of the pyruvate, the protein structure must be capable of sufficient flexibility so as to provide access of the substrate to the cofactor.

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Acknowledgments—We want to acknowledge the excellent technical assistance of Miss Hazel P. Williams and Mrs. Iris Turkenkopf.

The importance of at least one sulphydryl group for the enzymatic activity of pyruvate decarboxylase has recently been reviewed by Brauner and Ulirich (33). In addition, they showed that, of the four exposed sulphydryl groups (defined by their ready reactivity with N-ethylmaleimide), between one and two are protected in the presence of substrate or inhibitors.
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