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Acetoacetate Decarboxylase. Selective Acetylation of the Enzyme*

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ABSTRACT: Acetoacetate decarboxylase can be selectively acetylated with acetic anhydride or with 2,4-dinitrophenyl acetate to introduce one acetyl group for 60,000 mol wt units (two subunits). The acetylated enzyme is completely inactive. The site of the acetylation has been identified as the same lysine residue where a Schiff base with substrate is formed during enzyme decarboxylation. Acetoacetate decarboxylase can be heat activated. If the enzyme is not completely activated prior to acetylation, a partial heat reactivation of the acetylated enzyme can be achieved, but this does not involve loss or rearrangement of acetyl groups. A partial heat reactivation can be carried out for a similar enzyme into which an isopropyl group has been introduced by borohydride reduction of the enzyme–substrate reaction mixture.

The enzyme acetoacetate decarboxylase from *Clostridium acetobutylicum* catalyzes the decarboxylation of acetoacetate by way of a Schiff base intermediate formed between the enzyme and the substrate (Hamilton and Westheimer, 1959). The pathway for the reaction proceeds through the Schiff base of the product (acetone), and this Schiff base can be "trapped" by borohydride reduction, to yield a protein containing a residue of ϵ-*N*-isopropyllysine (Fridovich and Westheimer, 1962; Warren *et al.*, 1966). The protein obtained by this reduction of the Schiff base has been hydrolyzed with trypsin, and the peptide at the active site of the enzyme isolated, purified, and identified (Laursen and Westheimer, 1966); it proved to be Glu-Leu-Ser-Ala-Tyr-Pro-IprLys-Lys.[1] The enzyme is oligomeric (a dodecamer; see Tagaki and Westheimer, 1968) but only one of the lysine residues out of approximately 36 contained in two subunits (Lederer *et al.*, 1966) participates in the enzymic reaction.

Experiments on acetylation of the enzyme now show that the same lysine residue is especially active as a nucleophile; it readily attacks acetic anhydride or 2,4-dinitrophenyl acetate, and is thereby acylated. The resulting acetylated enzyme is essentially inert. The stoichiometry of the process has been established using radiochemically labeled acetic anhydride and by measuring, spectrophotometrically, the amount of 2,4-dinitrophenol formed from the reaction between the enzyme and 2,4-dinitrophenyl acetate. The evidence for the identity of the lysine residue in Schiff base formation and acetylation comes from the isolation and analysis of the peptides obtained by tryptic cleavage of the proteins obtained in these two processes. A complication arose because some samples of the acetylated enzyme can be partially reactivated by heat. Neece and Fridovich (1967) have now discovered that the enzyme itself can be heat activated; acetylation of enzyme that has previously been fully heat activated yields a product that cannot be heat reactivated.

Experimental Section

Materials. Crystalline acetoacetate decarboxylase was generally prepared by a modification of the method

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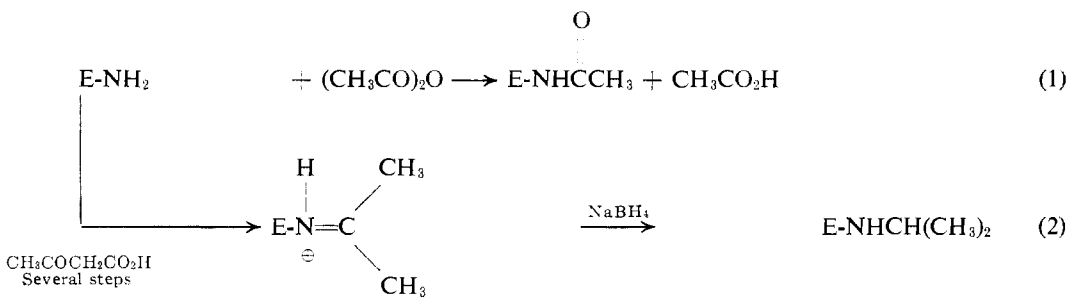
[1] Abbreviation used: IprLys, ϵ-*N*-isopropyllysine.



previously published (Zerner *et al.*, 1966; *cf.* Westheimer, 1968). The concentration of the enzyme is given in terms of subunits of mol wt 29,000 rather than in terms of the oligomer (Tagaki and Westheimer, 1968). Eight samples of the enzyme that had been dialyzed exhaustively against 0.05 M potassium phosphate buffer (pH 5.92) (to remove ammonium sulfate) were analyzed by the Kjeldahl–Nessler method by Dr. K. B. Taylor of the Biology Department, M.I.T., whose assistance we gratefully acknowledge. Based on 16.9% as the nitrogen content of the enzyme (calculated from its amino acid composition; *cf.* Lederer *et al.*, 1966), a solution with an optical density at 280 mμ of 1.00 in a 1.00-cm cell contains 0.95 ± 0.05 mg of the protein. This figure is slightly lower than the value of 1.00 mg previously assigned (Lederer *et al.*, 1966) for a solution of this optical density; all enzyme activities have been corrected to this new standard.

A sample of acetoacetate decarboxylase was prepared in the cold. Freshly prepared acetone powder of *Cl. acetobutylicum* was dried under vacuum at 0° for 3 hr and extracted with stirring overnight at 2° with 0.05 M phosphate buffer (pH 5.9). The solid was removed by centrifugation, and the regular preparative procedure continued except that all operations were conducted at 0–2°. In particular, the acetic acid precipitation was carried out at 0° to a pH of 4.05, determined with a Radiometer pH meter calibrated at 22°; the temperature compensator was set at 0° for the actual pH measurement.

Acetic anhydride had previously been carefully fractionated. Acetic anhydride-^{14}C (1.00 mCi/mmole) was obtained from the New England Nuclear Corp. Crude 2,4-dinitrophenyl acetate melted at 65–69° (Bender *et al.* (1966) report 71–71.5°). Trypsin that had been treated with L-tosylamido-2-phenylethyl chloromethyl ketone to inhibit chymotrypsin was purchased from Calbiochem. For other materials, commercial chemicals of high purity were used without further purification.

Methods. Radioactivity was measured with a Nuclear-Chicago Model 720 scintillation counter, using the scintillation solutions previously described (Warren *et al.*, 1966). The channels ratio method was used to monitor counting efficiency, which proved satisfactorily constant. Paper strips were counted on a Vanguard scanner that was kindly loaned to us by Professor P. M. Doty. Paper chromatography was conducted in a Chromatocab or in a large battery jar, using 20 × 50 cm Whatman No. 3MM sheets that had been washed with 7% acetic acid for at least 2 days prior to use.

Specific Activity of Acetic Anhydride-1-^{14}C. Duplicate 100-μl aliquots of the standard solution of acetic anhydride in dioxane were hydrolyzed in water at 35° for 10 min, and the resulting acid titrated to the phenol red end point. Comparison of the results of scintillation counting with the titration data showed that the specific activity of the acetic acid was 8.24×10^5 cpm/μmole.

Amino acid analyses were carried out with a Beckman–Spinco automatic amino acid analyzer (Model 120B) equipped for accelerated analysis. Prior to analysis, peptides were hydrolyzed for 22 hr in 6 N hydrochloric acid in sealed, evacuated ampoules at 107°. Samples for analysis usually contained 0.01–0.05 μmole of the major peptide.

Acetylation of Acetoacetate Decarboxylase. To a sample of 0.20–100 mg of acetoacetic acid decarboxylase in 1.00–15.0 ml of 0.1 M phosphate buffer, pH 5.9 at 0°, a solution of 10% acetic anhydride in dioxane (10 μl/ml of enzyme solution) was added and the mixture vigorously shaken. When acetic anhydride-^{14}C was used, the enzyme solution was dialyzed after the acetylation against two successive 1-l. portions of buffer. The enzyme for these latter experiments had a specific activity of only 35–36 units/mg.

Borohydride Reduction. To a stirred solution of 0.20 mg of acetoacetate decarboxylase in 1.00 ml of phosphate buffer (pH 5.95) were added simultaneously from two syringes 0.5 ml of 0.3 M lithium acetoacetate solution and 0.5 ml of cold 0.03 M sodium borohydride solution that was 0.001 M in sodium hydroxide. The addition was made in three parts, 15 sec apart. The solution was assayed within a few minutes.

Borate Inhibition. A solution of 0.1 ml of stock solution of acetoacetate decarboxylase (0.6 mg/ml) and 0.1 ml of sodium tetraborate (0.01 M) was incubated for 30 min at 0° and assayed in a solution of 1.45 ml of sodium tetraborate solution, 1.25 ml of pH 5.95 phosphate buffer, and 0.3 ml of 0.3 M lithium acetoacetate solution. No inhibition was observed. (Borate solutions contain various polymeric and hydrated borate anions, but they are all in rapid equilibrium with one another; *cf.* Ross and Edwards, 1967.)

In a second type of control experiment, 0.013 g of sodium borohydride was dissolved in 10 ml of phosphate buffer (pH 5.90) at 20°. At this pH the decomposition of the borohydride is very rapid. Subsequent to the decomposition of the borohydride, the solution was incubated with the same volume of a solution of acetoacetate decarboxylase (0.4 mg/ml), and then 0.1

ml of this solution was assayed in 1.5 ml of the borohydride decomposition solution, 1.1 ml of buffer, and 0.3 ml of lithium acetoacetate solution. The rate was 24% less than that of the control. The concentration of borate (0.017 M) was at least 30 times that present for the assays of borohydride-reduced enzyme–substrate mixtures.

Peptide Mapping. Samples of inactivated and heat-reactivated ^{14}C-acetylated acetoacetate decarboxylase were denatured and subjected to tryptic hydrolysis and two-dimensional peptide mapping as described by Lederer *et al.* (1966), *i.e.*, chromatography in butanol–acetic acid–water (4:1:5) followed by electrophoresis at pH 3.5 at right angles to the direction of chromatography. The two-dimensional maps were inspected both by autoradiography and by cutting the map into strips and scanning these strips with a strip scanner. One and the same radioactive spot was found on the peptide map, regardless of whether or not the acetylated acetoacetate decarboxylase had been reactivated by heating.

Isolation of the Peptide Ac-1. Acetic anhydride-1-^{14}C (1 mg) was added at 0° to a solution of 100 mg of acetoacetate decarboxylase dissolved in 12 ml of 0.1 M (pH 5.9) phosphate buffer. The mixture was shaken and allowed to stand at 0° for 0.5 hr. An additional 5 mg of nonradioactive acetic anhydride, dissolved in 50 μl of dioxane, was added, and the solution was left overnight at 0°. After a small amount of precipitate had been removed by centrifugation, the material was desalted at room temperature on a 2.5 × 100 cm column of Sephadex G-25 that had previously been equilibrated with 0.05 M ammonium bicarbonate solution. The fractions containing radioactive protein were combined and lyophilized. The solid residue was redissolved in 4 ml of 0.1 M ammonium hydroxide solution, denatured on a steam bath, and digested with trypsin according to the procedure of Laursen and Westheimer (1966).

The resulting mixture of peptides was first chromatographed on a 2.5 × 90 cm column of Sephadex G-25 fine, with elution with 0.05 M ammonium bicarbonate solution. The effluent from the column was led through a 2-ml radioactivity flow cell attached to the Nuclear-Chicago scintillation counter. The combined radioactive fractions were evaporated to 1.5 ml and applied to a column of Dowex 1-X2 that had been washed with 0.05 M hydrochloric acid, followed by 1.0 M sodium acetate, followed by 2.0 M acetic acid, followed by 1% 4-picoline–1% 2,4-lutidine acetate (pH 8.35). Elution was carried out with a linear gradient of 300 ml of 0.4 M acetic acid into 300 ml of the picoline–lutidine acetate buffer. The effluent was again surveyed for radioactivity with a flow cell. Samples (10 μl) of the fractions around the radioactive peak were taken for scintillation counting. The combined radioactive fractions were evaporated in a desiccator and redissolved in 1 ml of 2% aqueous pyridine. A 50-μl portion was hydrolyzed and subjected to amino acid analysis.

High-voltage electrophoresis was carried out using a Savant flat-plate water-cooled unit. Pyridine–acetate buffers were used at pH 3.5 and 6.5, and electrophoreses were run for 0.75–1 hr at 3000 V (75 V/cm). At pH 1.7, 1.5 M formic acid was used for 1 hr at 2220 V (55 V/cm). Wicks were cleaned by electrophoresis before use. Whatman No. 3MM paper which had been washed with 7% acetic acid for at least 2 days by descending chromatography was used for all electrophoreses. Paper strips cut from these sheets were surveyed on a Vanguard scanner, kindly loaned by Professor P. M. Doty. The radioactive areas were eluted by 2% pyridine solution.

Polyacrylamide Gel Disc Electrophoresis. Electrophoresis in polyacrylamide gels (Ornstein, 1964; Davis, 1964; Williams and Reisfeld, 1964; Richards *et al.*, 1965) was carried out with a Canalco apparatus.

Burst Titration of Acetoacetate Decarboxylase. Buffer (0.10 M phosphate buffer, pH 5.90) was introduced into a 3-ml cuvet of the Cary spectrophotometer and the base line at 360 mμ determined with the 0–0.1-OD slide wire. An aliquot of solution of 2,4-dinitrophenyl acetate (0.005 M in acetonitrile) was added and the "spontaneous" hydrolysis followed for several hundred seconds. An aliquot (100 μl) of stock solution of enzyme was added in the same buffer and the hydrolysis was followed; after the burst, the rate of increase in optical density again becomes equal to the "spontaneous" rate. Extrapolation of the spontaneous rate of hydrolysis of the substrate to the time of addition of the enzyme gives a direct measure of the burst. The calculations of concentrations from the results of burst experiments have been discussed by Bender *et al.* (1966). Corrections must be made for dilution on addition of the enzyme solution, for the time elapsed (here, about 25 sec) while the Cary is turned off during addition of the enzyme solution, and for the (slight) absorption of the enzyme at 360 mμ. The extinction coefficient for 2,4-dinitrophenolate ion–2,4-dinitrophenol solution at 360 mμ in pH 5.90 buffer at 30° was found to be 14,600 (*cf.* Bender *et al.*, 1966). The enzyme used in these experiments had previously been heat activated, and had a specific activity of 51 units/mg.

Results

Stoichiometry of the Acetylation. The stoichiometry of the acetylation has been measured in two ways: by the radioactivity of the acetylated enzyme and by the "burst" titration with 2,4-dinitrophenyl acetate. Acetylation of the enzyme with radioactive acetic anhydride led to the results shown in Table I.

The last column in Table I is entitled molar ratio Ac/subunit corrected. The correction takes into account the fact that the enzyme used in these experiments had not been heat activated; calculation of the ratio is based on the assumption that the molecular weight of the subunit is about 29,000 (Tagaki and Westheimer, 1968), and that the extent of reactivation on heating the acetylated enzyme (Figure 1) represents the extent to which further acetylation would be possible. The results of the "burst" titrations with 2,4-dinitrophenyl acetate are shown in Table II, and a typical experiment is illustrated in Figure 2.

TABLE I: Acetylation of Acetoacetate Decarboxylase with Radioactive Acetic Anhydride.[a]

Sample	Enzyme, Concn of Subunits ($\times 10^5$ M)	Catalytic Act. (units/mg)	Radioactivity (cpm/ml)	Ac/Subunit	Molar Ratio Ac/Subunit (cor; see text)
AAD	1.09, 1.41	37.2, 38.0	0, 0		
AcAAD	1.15, 1.45	0.0, 0.0	4510, 5490	0.50, 0.48	0.62
Reactivated AcAAD[b]	1.15, 1.45	10.7, *c*	4380, 5440	0.48, 0.48	0.56

[a] Abbreviations used: AAD, acetoacetate decarboxylase; AcAAD, acetylated acetoacetate decarboxylase. [b] Acetylated acetoacetate decarboxylase that had been partially reactivated by heating. [c] Not measured. Estimated as about 7 based on data such as those in Figure 3.

Site of Acetylation of Acetoacetate Decarboxylase. When acetoacetate decarboxylase, acetylated with acetic anhydride, was digested with trypsin, the peptides could partially be separated by chromatography. Analysis of the resulting mixture of peptides is shown in the first column of Table III. Although the analysis shows the amino acids previously found in the peptide at the active site (above the horizontal line), it shows many other amino acids as well. In particular, since the mixture contains both lysine and arginine, it is unlikely to represent a single peptide. Electrophoresis at pH 6.5, 3.5, and 1.7 did not produce pure peptide. Attempts to separate the peptides by acetylation followed by electrophoresis also failed. (These experiments were predicated on the assumption that the ϵ-amino group of the free lysine could be acetylated, whereas arginine could not, and that the resulting peptides would be electrophoretically separable.)

In duplicate experiments, descending paper chromatography in butanol–acetic acid–water (4:1:5) of the peptide mixture from the Dowex 1 column effected considerable purification. The radioactive peptide was located by strip scanning. In each case, the spot was cut out, the peptide was eluted with 2% pyridine and hydrolyzed, and the hydrolysate was subjected to amino acid analyses; the average of the results is shown in Table III. Although other amino acids have not been completely eliminated, and although in particular an appreciable quantity of glycine has been found, the major peptide corresponds in composition to Glu-Leu-Ser-Ala-Tyr-Pro-Lys-Lys, *i.e.*, to the peptide determined from borohydride reduction of the Schiff base from enzyme and substrate (Laursen and Westheimer, 1966).

Heat Activation of Acetoacetate Decarboxylase. The activation of acetoacetate decarboxylase by heating has been reported by Neece and Fridovich (1967). They observed a doubling of activity, from about 13 arbitrary units to about 26 units/mg; the

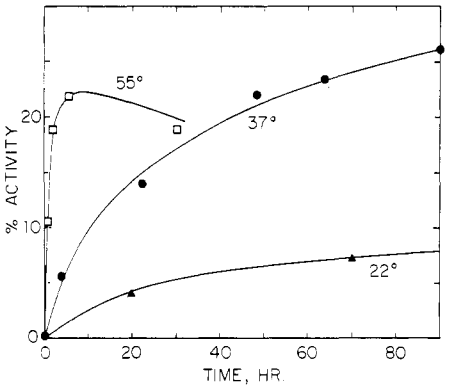


FIGURE 1: Reactivation of acetylated acetoacetate decarboxylase on heating at pH 5.95 in phosphate buffer. Concentration of enzyme 0.1–0.2 mg/ml (3.3–6.6 $\times 10^{-6}$ M in subunits). "Per cent activity" refers to percentage of initial activity of enzyme prior to acetylation (36 units/mg).

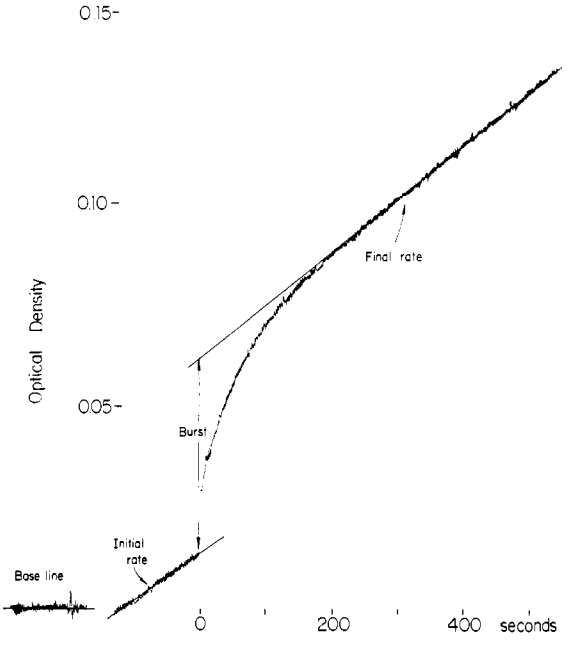


FIGURE 2: A "burst" titration of acetoacetate decarboxylase with 7.9×10^{-5} M 2,4-dinitrophenyl acetate.

TABLE II: Burst Titrations of Acetoacetate Decarboxylase with 2,4-Dinitrophenyl Acetate.[a]

Dinitrophenyl Acetate Concn (M)	Obsd Burst of Dinitrophenol (ODU)	Cor[b] Burst (ODU)	Burst (10^{-6} M)	Molar Ratio Ac/Subunit
1.6×10^{-5}	0.0482	0.0450	3.08	0.59
3.7×10^{-5}	0.0403	0.0387	2.65	0.50
3.7×10^{-5}	0.0478	0.0464	3.18	0.62
7.9×10^{-5}	0.0458	0.0451	3.09	0.59

[a] Conditions: pH 5.90 in 0.10 M phosphate buffer, 31°. The enzyme concentration was 5.17×10^{-6} M based on subunits of mol wt 29,000. The solvent contained 0.3–1.7% acetonitrile, introduced with the 2,4-dinitrophenyl acetate. [b] Corrected for the dilution on addition of enzyme solution and for absorption of enzyme at 360 mμ.

specific activity of their enzyme is thus considerably lower than that of ours (35–55 units/mg). Our preparation of enzyme in the cold led to inefficient chromatography, and thus to impure enzyme; the best fraction had a specific activity of only 9.5 units/mg, but was increased on heating at 55° (for about 3 hr or for 7 hr) to about 30 units/mg. Part of this increase in specific activity represents an increase in purity, as inactive protein is denatured and removed, but most of the increase is real, and is accompanied by a 70% increase in total activity.

Much of the enzyme used in the present study was prepared according to Zerner *et al.* (1966) without specific heat activation. Such enzyme usually had a specific activity from 35 to 50 units/mg; since it had been prepared at room temperature and above, most of the heat activation had already occurred during the preparation. Although such enzyme was crystalline, and although its activity considerably exceeds that of the heat-activated enzyme of Neece and Fridovich, it could still be further activated (usually by about 20–30%) on heating for several hours at 55°.

When enzyme, prepared without specific heat activation, was acetylated, completely inactive material was obtained. However, on heating, this acetylated enzyme partially regained activity. The reactivation at pH 5.95 is shown for various times in Figure 1 for enzyme of initial specific activity of 35–36 units/mg; reactivation on heating at other pH values is similar. After the acetylated enzyme has been heat activated, reacetylation again destroys activity, and then only a few per cent of activity can be recovered by further reheating. When a sample of enzyme was heated for 6 hr at 55° prior to acetylation, less than 3% of activity could be regenerated on heating the acetylated product.

Succinylation of the Enzyme. Acetoacetate decarboxylase that has not specifically been heat activated is inactivated by succinic anhydride very much as by acetic anhydride, and the enzyme reactivated to about the same extent on heating. No further reactivation is achieved after about 4-hr heating at 55°; about 20% of the original enzymic activity was recovered. The succinylated enzyme gives only a single band on analytical acrylamide gel electrophoresis, and the succinylated material can be readily separated from mixtures with native enzyme by this technique.

Activation of the Isopropylated Enzyme on Heating. In earlier experiments (Fridovich and Westheimer, 1962; Warren *et al.*, 1966) enzyme and substrate were reduced with sodium borohydride. The reaction led to the isopropylation of an essential lysine in the enzyme as a result of reducing the Schiff base salt of the product, acetone (see eq 2). The reaction was accompanied by a large but incomplete inactivation of the enzyme; even on repeated reductions, it retained 3–8% of its activity. Although complete loss of enzymic activity on converting a lysine residue to an alkyl lysine residue is not absolutely demanded by the mechanism, it had been anticipated that inactivation would be complete. It has now been found that borohydride reduction does indeed lead to complete inactivation when the assay is performed immediately; furthermore, borate ion is not

TABLE III: Partial Purification of Acetyl Peptide.

Amino Acid	Material from Dowex	After Paper Chromatography[a]	Peptide at Active Site[b]
Glu	(1.00)	(1.00)	1
Leu	1.03	1.09	1
Ser	0.36	0.83	1
Ala	1.04	1.23	1
Tyr	0.49	0.71	1
Pro	0.92	1.15	1
Lys	2.38	2.03	2
Arg	0.09		
Asp	0.52	0.26	
Thr	0.19		
Gly	0.17	0.62	
Val	0.36	0.07	
Ile	0.47	0.11	

[a] Average of three experiments. [b] Laursen and Westheimer (1966).

TABLE IV: Reactivation of AAD Inactivated by Sodium Borohydride Reduction in the Presence of Acetoacetate.

Expt	Time at 55° (hr)	Assay: Change in OD/sec × 10^5 [a]	Act (%)
1[b]	0	0	0
	1.0	20	9.0
	2.0	28	13
2[c]	0	0	0
	0.50	8.0	7.0
	1.25	14	13
	1.80	18	16
	3.00	19	17

[a] All borohydride was destroyed by acid-catalyzed decomposition in the buffer prior to assay. [b] Final concentration of solution for reduction was 0.075 M in acetoacetate and 0.012 M in $NaBH_4$. [c] Final concentration was 0.02 M in acetoacetate and 0.003 M in $NaBH_4$.

an inhibitor, at least in the concentrations in question, as are many other anions (Fridovich, 1963). The incomplete inactivation previously observed is presumably a result of the partial reactivation of the isopropylated enzyme on standing at room temperature.

In particular, the isopropylated enzyme when prepared from "ordinary" acetoacetate decarboxylase (*i.e.*, when prepared from enzyme that has a specific activity of about 40 units/mg, but which has not been specially activated by heating) can be reactivated by heating to about the same extent as the corresponding acetylated enzyme. The appropriate data are shown in Table IV.

Discussion

The data here presented show that acetoacetate decarboxylase reacts quantitatively and selectively with acetic anhydride or with 2,4-dinitrophenyl acetate to acetylate the enzyme with complete loss of enzymic activity. A partial reactivation on heating the acetylated enzyme occurs only with enzyme that has not previously been fully heat activated. Furthermore, as shown in Table I, such samples of acetylated enzyme do not lose ^{14}C when they are partially reactivated by heat; the reactivation process is then concerned with the structure or conformation of the enzyme and not with a loss of acetyl groups. Mapping of the tryptic peptides from the heat-activated acetylated enzyme shows that only the same radioactive acetyl peptide is formed as before heat treatment; the acetyl group has neither been removed nor relocated.

The stoichiometry of the process shows that one acetyl group per two subunits is sufficient to inactivate the enzyme. It has not yet been established, however, whether two subunits constitute one active site, or whether half of the subunits are inactive or hidden.

The isolation of the peptide produced on acetylation makes it reasonably sure that it is the same as that which is isopropylated in the borohydride reductions in the presence of substrate. The Sephadex and Dowex 1 chromatographies do not lead to a pure peptide, and the contaminating peptide is extremely difficult to separate from the acetylated material. However, paper chromatography of the impure peptide from the Dowex column yielded a material that was substantially identical in composition with the peptide previously identified as that at the active site. The only major contaminant in the peptide is glycine, and this material is nearly ubiquitous and frequently is found as a contaminant in elution of paper (Canfield and Anfinsen, 1963).

Furthermore, the lysine residue that serves as the active site for the native enzyme has here been acetylated. That a lysine has been acetylated follows from the isoelectric point of the peptide (near pH 6.5); it must then have equal numbers of positive and negative charges. This conclusion is confirmed (and the location of the acetyl group on the penultimate lysine established) by the known specificity of trypsin, which otherwise would have cleaved the Lys–Lys bond of the peptide.

Two questions remain with respect to the work here presented. (a) Although the enzyme is crystalline and homogeneous to ultracentrifugation and gives only a single band on analytical acrylamide gel electrophoresis, it may nevertheless be impure. This doubt arises from the results of chromatography over dimethylaminoethylcellulose, where the variation in specific activity among the fractions exceeds experimental error. Although these variations are not large, they cast doubt on the complete purity of the enzyme. While the authors do not believe that the amounts of impurities can be sufficient to upset the proposed stoichiometry (two subunits for the active site), the question cannot be regarded as completely settled. (b) The mechanism of heat activation is not yet known.

Acknowledgments

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Monodispersity and Quaternary Structure of Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: The molecular weight of glyceraldehyde 3-phosphate dehydrogenase (GPD) has been reported to be in the range between 117,000 and 150,000 suggesting a dissociation–association equilibrium between the native enzyme and its protomers. To check this hypothesis the monodispersity of yeast and rabbit muscle GPD has been estimated comparing the mean values of M from sedimentation analysis, light scattering, and osmotic pressure. On the other hand, the influence of pH, ionic strength, solubilizing additives, etc., on the dissociation behavior has been investigated to analyze the interprotomer forces stabilizing the native quaternary structure.

The results confirm the molecular weight of 144,700 ± 3000 in accordance with a tetrameric quaternary structure. No anomalous dependence on protein concentration or temperature is observed in the entire pH range of enzymic activity. The values for the weight, number, and s,D average of M are identical within the limits of error. The enzyme, therefore, may be assumed to be strictly monodisperse. It is evident from ultraviolet differences pectra, opticalr otatory dispersion, and circular dichroism that dissociation into subunits in principle is accompanied by conformational changes. Therefore, no distinct indication regarding the interprotomer binding sites can be given. Qualitatively, the dissociation parameters lead to the conclusion that ion pairs and hydrogen bonds predominate as association sites while hydrophobic interactions seem to be of minor importance.

Glyceraldehyde 3-phosphate dehydrogenase (GPD,[1] EC 1.2.1.12) from rabbit skeletal muscle has been reported to have a molecular weight between 118,000 and *ca.* 150,000 (using sedimentation analysis and light scattering as analytical methods) (Dandliker and Fox, 1955; Fox and Dandliker, 1956; Taylor and Lowry, 1956; Elödi, 1958; Elias *et al.*, 1960).

Recently Jaenicke (1963) and Harrington and Karr (1965) found strong physicochemical evidence for an M value in the range of 140,000, verifying the figures of Dandliker and Fox (1955), Fox and Dandliker (1956), and Elödi (1958) and the "chemical" molecular weight of 140,000 following from amino acid analysis and end-group analysis as well as peptide mapping (Harris and Perham, 1965). The broad range of variability even under similar conditions of the experiments suggests a solvent- or concentration-dependent equilibrium

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[1] Abbreviations used: BSA, bovine serum albumin; Y-GPD, yeast glyceraldehyde 3-phosphate dehydrogenase; R-GPD, rabbit muscle glyceraldehyde 3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; NAD, nicotinamide–adenine dinucleotide.