Studies on the Presence and Role of Tryptophan in Pig Heart Mitochondrial Malate Dehydrogenase*

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TIN-LOK CHAN† AND KARL A. SCHELLENBERG§

From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

SUMMARY

Several different experimental approaches have shown the presence of tryptophan in malate dehydrogenase from pig heart mitochondria. The methods used were (a) fluorescence measurement of tryptophan content after acid hydrolysis and separation of amino acids by paper chromatography, (b) recovery of tryptophan labeled with tritium after incubation of the enzyme with tritiated substrates, (c) determination of tryptophan content of acid and alkaline hydrolysates with the amino acid analyzer and (after silylation) by gas chromatography, (d) the ultraviolet spectral method of Edelhoch (Biochemistry, 6, 1948 (1967)), and (e) determination of tryptophan by reaction with 2-hydroxy-5-nitrobenzyl bromide. The estimation of tryptophan by fluorescence, tritium-labeling, the amino acid analyzer, or gas chromatography of acid or alkaline hydrolysates usually indicated 0.3 to 1.1 residues per molecule of enzyme, whereas the two nonhydrolytic methods (ultraviolet absorption and reaction with 2-hydroxy-5-nitrobenzyl bromide) indicated about 2 tryptophanyl residues per molecule of enzyme.

The protein acquires about 0.5 atom of tritium per molecule of enzyme from labeled substrate under conditions similar to the previously reported labeling of yeast alcohol dehydrogenase and rabbit muscle lactate dehydrogenase. The positive results in three different DPN-linked enzymes suggest that in some members, at least, of this class of enzymes dehydrogenation of an enzyme tryptophanyl residue takes place through the intermediacy of an indolenine or equivalent structure.

The structure of the adduct of 2-hydroxy-5-nitrobenzyl bromide with D-methylindole was determined as a model for the primary adduct of the reagent with protein tryptophanyl residues.

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for enzyme activity. The enzyme peak appeared in Fraction 5 or 6; this peak fraction was lyophilized and solutions of the lyophilized enzyme in 0.1 M pyrophosphate buffer, pH 8.6, were used in labeling experiments. The specific activity of the unpurified enzyme was determined to be 417 units per mg when the initial rate of oxidation of DPNH was measured at 25° with the following final concentrations: potassium phosphate, pH 7.5, 0.1 M; oxalacetate, 50 μM; and DPNH, 200 μM. The activity was unchanged after DEAE-cellulose chromatography and lyophilization. Concentrations of native and denatured protein were routinely determined by ultraviolet absorption; for native enzyme ε₁₅₀ was 33,200, and for denatured enzyme in 0.2 M NaOH ε₁₅₀ was 54,000. These values were based on dry weight determination and a molecular weight of 70,000 (5).

α-L-Malate-2-¹'H—This substrate was prepared by borohydride reduction of oxalacetate (9). The labeled malate had a specific activity of 8.35 mCi per mmole when prepared from NaBH₄ with specific activity of 12.3 mCi per mmole. The radiochemical purity was checked by paper chromatography, and by similarity in labeling by undiluted material and that diluted 20-fold with unlabeled sodium DL-malate.

Electrophoresis—Cellulose acetate electrophoresis of the enzymes was carried out with the Gelman apparatus, with Sephracore III strips, 0.1 M Na₂HPO₄ buffer, pH 9, and at 200 volts across 12 cm for 1½ hours. Protein, 10 to 50 μg, was applied to each strip. After electrophoresis the strips were stained for protein (10) and in some cases strips were cut lengthwise and one portion was stained for protein and another for enzyme activity. In the latter case the lactate dehydrogenase assay was adapted by substitution of l-malate for lactate (11).

Paper Chromatography—Descending chromatography with the system 1-propanol-water-ammonium hydroxide, 55:30:10, with Whatman No. 1 paper was carried out for 19 hours at room temperature. Under these conditions Rf values were approximately as follows: malate, 0.52; lactate, 0.73; tyrosine, 0.69; tryptophan, 0.75; and tryptophan methyl ester, 0.95.

Florisil Chromatography—Radiochemical purification of N-acetyl-νL-tryptophan was accomplished by chromatography on a column (0.6 × 16 cm) of Florisil (60 to 100 mesh, Floridin Company, Warren, Pennsylvania). The material, 10 mg, in 20 ml of ethyl acetate, was applied to the column prepared in ethyl acetate. Fractions were successively eluted with ethyl acetate ethanol, ethanol-acetic acid, and glacial acetic acid. N-Acetyl-νL tryptophan was sharply eluted in the 1:10 acetic acid ethanol fraction, whereas tryptophan was eluted in the glacial acetic acid, and acetyltryptophan methyl ester in ethyl acetate.

Gradient Ion Exchange Chromatography of Tryptophan—An adaptation of the technique of Moore and Stein (12) was used to determine the radiochemical purity of ²H-labeled tryptophan derived from malate dehydrogenase. Tryptophan-³C was added to ²H-labeled hydrolysate, and the ²H:³C ratio was determined in fractions after chromatography. The sample containing 9.3 mg of tryptophan in 2 ml of ethyl-water, 1:1, was applied to a column, 0.12 × 16 cm, of Dowex 50W-X8. The column was eluted with 500 ml of a linear gradient from 0.1 M ammonium acetate, pH 4.7, in 50% 2-propanol, to 0.5 M ammonium acetate, pH 5.2, in 50% 2-propanol. Fractions of 5 ml were counted in both ²H and ³C channels, and the absolute activity of ²H and ³C in each sample was calculated with the aid of external and added internal standards.

Fluorescence Studies The λₐₘₙ of activation and emission were determined manually or from the recorded spectra, with the use of an Amino-Bowman spectrophotofluorometer. The 1P-28 photomultiplier and slit combination No. 3 were used. The wave length scales were calibrated with the mercury lines from a Pen-Ray lamp. The precision of wave length measurements was about ±5 nm. Fluorescence assay of paper chromatograms was done by cutting the chromatograms into 1-cm segments, eluting the segments with ethanol-water, 1:1, and taking the spectra of the eluates.

Radioactivity Determinations—Tritium was assayed with a Beckman LS-100 liquid scintillation counter. A mixture of dioxane-naphthalene-diphenyloxazole (180:20:1) was used as a scintillation solution, with the addition of methanol or ethanol for polar aqueous solutions, and tetramethylammonium hydroxide and dithioglycol for tryptophan and derivatives. The tritium counting efficiency was 20 to 30%, and background was 15 to 30 cpm. Paper chromatograms were counted after shaking 1-cm segments in the counting vial with 2 ml of scintillation solution-methanol (10:1).

Tryptophan Determination after Method of Edelbach (13)—The ultraviolet spectra of the various proteins in 6 M guanidinium chloride and 0.02 M phosphate, pH 6.5, were taken with the Bausch and Lomb 505. The wave length scale was calibrated with the built-in mercury lamp. Ultraviolet-absorbing impurities in the guanidinium chloride were removed by treatment with Norit and the equation (10)

\[ N_{T_{p}} = (t_{280}/3,103) - (t_{280}/10,318) \]

was used directly after extrapolation of the 310 to 400 nm baseline to the wave lengths measured. No correction for possible cystine was made. Calculated values of \( N_{T_{p}} \) were practically the same when determinations were repeated with a 2-fold variation in protein concentration.

Tryptophan Analysis after Acid Hydrolysis—Malate dehydrogenase, and for comparison, yeast alcohol dehydrogenase, were hydrolyzed in 6 M HCl for 8 hours at 112° in evacuated sealed ampoules. Following removal of HCl by lyophilization, tryptophan and arginine were determined with the amino acid analyzer. Tryptophan was also determined in aliquots of the hydrolysates by gas chromatography of the silyl derivative as described below.

Tryptophan Analysis after Alkaline Hydrolysis—The proteins, 1 to 10 mg, and, as controls, N-acetyltryptophan and tryptophan, were hydrolyzed in a mixture of 1 ml of water and 180 mg of Ba(OH)₂ in H₂O for 17 hours at 112°. After hydrolysis the base was neutralized by addition of 0.5 ml of 4 M NaH₄PO₄. The amino acids were then extracted with 4 ml of 1-butanol. Following lyophilization of the butanol, tryptophan was analyzed with the amino acid analyzer and by gas chromatography. Control experiments indicated that 67% of added tryptophan was extracted into the butanol under these conditions. N-Acetyltryptophan and tryptophan, after hydrolysis, gave the same peak area on gas chromatography, indicating complete hydrolysis, at least of the tryptophan N-amide bond.

Gas Chromatographic Analysis of Silylated Amino Acids (14, 15)—To the dry hydrolysate, 1 to 10 mg, was added 0.1 ml of bis-(trimethylsilyl)-trifluoroacetamide (Regis Chemical Company, Chicago). The tube was sealed and incubated for 1 hour at 127°. Between 2 and 10 μl were injected into the column. The Hewlett-Packard 700 instrument with flame ionization detector was used. Chromatograms were run at a temperature of 205°, with helium pressure at 50 p.s.i. Columns (4 inch × 6 feet)
were 10% silicone rubber on firebrick. Under these conditions the retention time of the tryptophan silyl derivative was 94 min, and the tryptophan silyl peak was well separated from other amino acid derivatives. Retention times of silyl derivatives of phenylalanine and tyrosine were 1.56 and 3.9 min, respectively. The tryptophan was quantitatively determined from the peak area. Tryptophan and phenanthrene controls were included.

Tryptophan Determination with 2-Hydroxy-5-nitrobenzyl Bromide—The procedure described by Barman and Koshland (16) was carried out with the following changes: (a) the protein was incubated for 1 hour at 25° with 8 M urea and 0.5 M sodium citrate buffer, pH 3.0, before addition of reagent. (b) the protein concentration in the Sephadex column eluate was determined from dry weight.

RESULTS AND DISCUSSION

Identity and Purity of Enzyme—Comparison of ultraviolet absorption, fluorescence (activation λ_{\text{max}} 275 nm and emission λ_{\text{max}} 306 to 307 nm in Fractions 1 to 10), and activity of the mitochondrial enzyme after DEAE-cellulose chromatography are illustrated in Fig. 1. In the experiment shown, Fraction 5 was lyophilized and used for some of the other experiments described below. In several other similar purifications the ratio of enzyme to DEAE-cellulose was varied 10-fold without change in fluorescence properties or enzyme activity, indicating an apparently complete separation from fluorescent impurities in the commercial product by this procedure.

The possible presence of DPN, DPNH, or substrates bound to the enzyme was sought by fluorometric determination of DPNH. Thus malate dehydrogenase, 10^{-4} M at pH 9.8, exhibited no fluorescence (λ_{\text{excitation}} 340 nm, λ_{\text{emission}} 450 nm) either alone or after addition of DPN or malate. Controls with enzyme plus malate and DPN, or enzyme plus DPNH, exhibited the characteristic fluorescence with intensity proportional to the concentration of DPNH either added or resulting from enzymatic action. The sensitivity was such that 0.03 molecule of DPNH, DPN, or malate per molecule of enzyme would have been detected. Coenzymes or substrate were thus either absent or present in very small quantities in the enzyme preparation.

Separation of the mitochondrial and supernatant enzymes of electrophoresis is shown in Fig. 2, and electrophoretic homogeneity of the mitochondrial enzyme is further indicated by the coincidence of protein and enzyme activity peaks (Fig. 3).

Purity of DL-Malate-2-^3H—Comparison of activity as substrate and radiochemical purity of synthetic labeled malate is shown in Fig. 4. In other experiments not shown, the labeled DL-malate, 15 mM, was incubated for 4 hour at 25° with malate dehydrogenase, 10^{-4} M, lactate dehydrogenase, 10^{-4} M, DPN, 0.7 mM, DL-lactate, 140 mM, and Tris-chloride, pH 8.1, 14 mM, and then similarly chromatographed. The tritium label was found in malate and lactate. Control experiments with single omission of the enzymes or DPN indicated that the labeled malate transferred tritium enzymatically via DPN to lactate.

Presence of Tryptophan in Mitochondrial Malate Dehydrogenase—Although DEAE-cellulose-purified enzyme showed fluorescence due only to tyrosine, the presence of tryptophan in such enzyme was established by several independent methods.

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**Fig. 1.** Mitochondrial malate dehydrogenase, 2 mg, in 5 ml of 0.01 M Tris-chloride, pH 8.1, was placed on a DEAE-cellulose column, 2 X 8.5 cm, previously equilibrated and eluted with the same buffer. Ultraviolet and fluorescence spectra were taken on 5-ml fractions. Enzyme activity was measured here as the initial Δ A_{240} per min, with final concentrations as follows: fraction diluted 1:200; potassium glycinate, pH 9.8, 50 mM; DPN, 7 mM; sodium L-malate, 50 mM. All work was done at 25°. A_{240}, ●.

**Fig. 2.** Cellulose acetate electrophoresis of pig heart mitochondrial malate dehydrogenase (M - MDH), supernatant (S - MDH), and a mixture of the two (M - + S - MDH).
substrate and coenzyme, was hydrolyzed to amino acids. A portion of the hydrolysate was chromatographed, and the correlation of tritium and fluorescence is shown in Fig. 5. For comparison, a mixture of tyrosine and tryptophan (20:1) was similarly chromatographed. It is seen that the main $^3$H peak coincides with the tryptophan fluorescence peak. (The nature of the second smaller radioactive peak is unknown; it may be a breakdown product of the acid hydrolysis of tryptophan.) Comparison of the ratio of the tryptophan to tyrosine fluorescence intensities in the hydrolysate with that in the 20:1 mixture indicates roughly a tryptophan to tyrosine ratio in the hydrolysate of 0.084. Assuming 12 tyrosines per molecule (4, 17), the hydrolysate contained about 1 tryptophan per molecule or 0.5 per subunit. If one further estimates that about half of the tryptophan was destroyed by acid hydrolysis, then the protein contained 2 tryptophans per molecule. Such comparisons give only a rough estimate, since they omit uncertainty in the amount of destruction by acid hydrolysis, quenching effects of other amino acids, nonlinearity of fluorescence to concentration ratio, incomplete elution, etc.

Crystallization of a similar acid hydrolysate with carrier L-tryptophan, and study of derivatives prepared therefrom,
indole, indicated that the tritium was in the methylene group of tryptophan (Table I). In several experiments the protein radioactivity varied from 0.30 to 0.75 atom of tritium per molecule, and of the total 20 to 50% was found in tryptophan after acid hydrolysis. Mitochondrial malate dehydrogenase, which must contain very little tryptophan, is thus labeled to practically the same extent as the tryptophan-rich yeast alcohol dehydrogenase and lactate dehydrogenase (1, 3). This similarity in stoichiometry not only is consistent with the previously postulated role of tryptophan in hydrogen transfer, but would also imply that the labeling reaction is specific for certain tryptophanyl residues at the active site in the other enzymes.

Application of the recent ultraviolet spectral method of Edelhoch for determination of tryptophan indicated about 1.8 residues per molecule, as shown in Table II. Yeast alcohol dehydrogenase, which contains about 28 tryptophans per molecule, and ribonuclease, which is devoid of tryptophan, served as controls.

The calorimetric determination based on reaction with 2-

![Graph](image)

**Fig. 6.** Gradient ion exchange chromatogram of a mixture of hydrolysate of 3H-labeled malate dehydrogenase and 14C-tryptophan. See "Experimental Procedure" for details. 3H, ○—○; 14C, ○—○.

**Table I**

**Position of tritium in enzyme**

The denatured protein was prepared and hydrolyzed by a method similar to that described in Fig. 5, except that hydrolysis time was 13 hours. The total radioactivity in the protein was equivalent to 0.48 atom of tritium per molecule. Carrier L-tryptophan was added to the hydrolysate and recrystallized, and derivatives were prepared therefrom.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Recrystallization</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denatured protein</td>
<td>3 times</td>
<td>10.00</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td></td>
<td>5.04</td>
</tr>
<tr>
<td>L-Tryptophan methyl ester hydrochloride</td>
<td>2</td>
<td>3.44</td>
</tr>
<tr>
<td>N-Acetyl-DL-tryptophan</td>
<td>2</td>
<td>4.90</td>
</tr>
<tr>
<td>N-Acetyltryptophan after Florisil</td>
<td>1</td>
<td>5.00</td>
</tr>
<tr>
<td>Indole</td>
<td></td>
<td>0.00</td>
</tr>
</tbody>
</table>

*Paper chromatography indicated that the tryptophan methyl ester was radiochemically pure.*

**Table II**

**Tryptophan determination after method of Edelhoch (15)**

Tryptophan was calculated from $A_{155}$ and $A_{280}$ as detailed in the text. Yeast alcohol dehydrogenase and pancreatic ribonuclease were from Boehringer.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (N$_{TP}$ residues per molecule)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate dehydrogenase</td>
<td>0.90 1.78</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>1.80 1.80</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>2.86 2.71</td>
</tr>
<tr>
<td>Yeast alcohol dehydrogenase</td>
<td>0.15 25.8</td>
</tr>
<tr>
<td>Yeast alcohol dehydrogenase</td>
<td>0.20 22.5</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>10.0 0.07</td>
</tr>
</tbody>
</table>

* After purification by DEAE-cellulose chromatography.  
As purchased, without further purification.

hydroxy-5-nitrobenzyl bromide (see "Experimental Procedure") indicated 2.0 tryptophanyl residues per molecule of malate dehydrogenase. In order to gain some insight into the mechanism of reaction and possible structure of the nitrophenyl-tryptophanyl adducts, 3-methylindole was studied as a model compound for protein tryptophanyl residues. A single crystalline product was obtained in several preparations with variations in procedure as noted. 3-Methylindole, 131 mg (1 mmole), was dissolved in 1 to 5 ml of methanol. 2-Hydroxy-5-nitrobenzyl bromide, 250 to 465 mg (1.1 to 2.0 mmole), was added; the resulting yellow solution had a pH of about 2. One milliliter of 2 M aqueous sodium acetate was added, and the yellow suspension, pH 5, was centrifuged, and the solid recrystallized from methanol or ethanol. The yields of crude product were quantitative, and after two recrystallizations were from 161 to 187 mg (57 to 66%), m.p. 157-159° (corrected); infrared $\nu_{max}$ 3,420 (sharp), 1,620, 1,590, 1,490 cm$^{-1}$; ultraviolet $\lambda_{max}$ 303 nm (e 9,000), $\lambda_{max}$ H$_2$O 264 nm (e 7,000), 414 nm (e 18,500); nuclear magnetic resonance (CDCl$_3$) $\delta$ 1.47 (singlet, 3 H), 3.10 (doublet split 2.5 cycles, 2 H), 4.15 (broad singlet, 1 H), 5.54 (singlet, 1 H), 6.5 to 8.0 (multiplets, 7 H). These peaks correspond to the methyl, methylene, NH, indoline CH, and aromatic protons, respectively. The methylene protons are split because of their differing relationships to the neighboring asymmetric carbon of the indoline.

C$_{10}$H$_{12}$N$_2$O$_3$

Calculated: C 68.07, H 5.00, N 9.93

Found: C 68.25, H 5.17, N 9.72

The absence of OH bands and presence of the NH band at 3,420 cm$^{-1}$ in the infrared spectrum indicate that the indoline NH is intact and the phenol OH is absent in the neutral compound. The infrared, nuclear magnetic resonance, and ultraviolet spectra and elemental analysis of the neutral compound require the tetracyclic structure shown below, 2-nitrobenzopyrano-(2,3b)-10b-methyl-indoline. The ultraviolet spectrum of the compound in ethanol ($\lambda_{max}$ 303 nm) is in agreement with the ring structure, but the strong yellow color in aqueous alkali ($\lambda_{max}$ 264, and 414 nm) indicates the formation of a p-nitrophenolate anion. Such ionization would be accompanied by ring opening...
The crystalline material is evidently a racemic mixture in which the configuration at the indole position 2 bears a fixed relation to that at C 3. If C 2 and C 3 were independently asymmetric, a noncrystalline mixture of diastereomers would have been obtained.

Since this work was submitted, Spande, Wilchek, and Witkop (18) have confirmed the structure of the 3-methylindole adduct. These workers have also shown a possible further alkylation at the indole nitrogen in certain derivatives of tryptophan, and therefore one must be cautious in the extrapolation of the reaction with simple model compounds to that with protein tryptophanyl residues.

The spectrophotometric method of Edelhoch and the reaction with 2-hydroxy-5-nitrobenzyl bromide gave values of about 2 tryptophanyl residues per molecule of enzyme. If the malate dehydrogenase is composed of two identical subunits (19-21), then the enzyme would contain 2 tryptophans per molecule or 1 per subunit. This value is consistent with the above estimate from fluorescence measurements after hydrolysis and chromatography. The tritium-labeling experiments are more ambiguous regarding the exact number of tryptophanyl residues; but they unequivocally establish the presence of tryptophan in the enzyme.

The presence of tryptophan was also confirmed with the amino acid analyzer, following alkaline and acid hydrolysis of the protein (see "Experimental Procedure"). Determination after alkaline and acid hydrolysis indicated 0.92 and 0.29 tryptophanyl residue per molecule of malate dehydrogenase, respectively. Amino acid analysis of yeast alcohol dehydrogenase after acid hydrolysis indicated 6.6 tryptophans per molecule or 1 per subunit. This value is consistent with the above estimate from fluorescence measurements after hydrolysis and chromatography. The tritium-labeling experiments are more ambiguous regarding the exact number of tryptophanyl residues; but they unequivocally establish the presence of tryptophan in the enzyme.

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**Table III**

**Tritium-labeling of protein**

Malate dehydrogenase (unpurified), 28.5 μM; potassium glycinate, pH 9.8, 0.1 M; and water or inhibitors as noted in the table, in a volume of 0.2 ml, were incubated for 10 min at 0°, and then added to a 10-min previously incubated mixture of yeast alcohol dehydrogenase, 0.1 μM; malate dehydrogenase, 0.1 μM; phosphate, pH 7.8, 0.2 M; sodium hydroxide, 1 mM; DPN, 1 mM; DPNH, 0.2 M; and L-malate, 25 mM in a volume of 0.2 ml. The mixture, final volume, 0.5 ml, was incubated for 10 min more at 0°, then 70% HClO4, 0.1 ml, was added. After NaOH or heat denaturation, and malate addition where indicated, the protein was precipitated with 2 M HClO4, and then treated as given in Table III. Final protein yields were 35 to 70%.

<table>
<thead>
<tr>
<th>Experiment and changes in conditions</th>
<th>Amount of tritium in protein</th>
<th>atom/molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>HClO4</td>
<td>0.44</td>
</tr>
<tr>
<td>2. Unlabeled malate after HClO4</td>
<td>HClO4</td>
<td>0.32</td>
</tr>
<tr>
<td>3. Unlabeled malate before HClO4</td>
<td>HClO4</td>
<td>0.01</td>
</tr>
<tr>
<td>4. None</td>
<td>Heat</td>
<td>0.46</td>
</tr>
<tr>
<td>5. Unlabeled malate after heat</td>
<td>Heat</td>
<td>0.52</td>
</tr>
<tr>
<td>6. None</td>
<td>NaOH</td>
<td>0.30</td>
</tr>
<tr>
<td>7. Unlabeled malate after NaOH</td>
<td>NaOH</td>
<td>0.33</td>
</tr>
</tbody>
</table>

**Table IV**

**Effect of method of denaturation on protein-labeling**

Enzyme was labeled by L-malate-2-H generated in situ as shown in legend to Table III. Protein was denatured as noted by addition of 2 M HClO4 by heating for 15 min at 100°, or by the addition of 1 M NaOH. Where indicated, unlabeled L-malate, 0.1 M, was added. After NaOH or heat denaturation, and malate addition where indicated, the protein was precipitated with 2 M HClO4, and then treated as given in Table III.

**Table V**

**Protein-labeling with synthetic DL-malate-2-H**

Malate dehydrogenase, 28.5 μM; potassium glycinate pH 9.8, 0.3 M; DPN, 10 mM; and DL-malate-2-H, 20 mM, in 0.5 to 5 ml were incubated for 10 min at 0°, and then protein was precipitated with 2 M HClO4 and treated as given in Table III. Average values and standard error of the mean are shown for experiments done at least twice.

<table>
<thead>
<tr>
<th>Experiment and changes in conditions</th>
<th>Amount of tritium in protein</th>
<th>atom/molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>HClO4</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>2. DEAE-cellulose-purified enzyme</td>
<td>HClO4</td>
<td>0.48 ± 0.14</td>
</tr>
<tr>
<td>3. Enzyme first heated for 10 min at 100°</td>
<td>HClO4</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>4. Phosphate, pH 7.8, in place of glycine</td>
<td>HClO4</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>5. Omit DPN</td>
<td>HClO4</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>6. Malate-2-H diluted 1:20 with unlabeled DL-malate</td>
<td>HClO4</td>
<td>0.29a</td>
</tr>
</tbody>
</table>

a Calculation based on a specific activity 5% of that of undiluted DL-malate-2-H.

molecule. Acid hydrolysis thus destroys about 70 to 80% of the tryptophan in yeast alcohol dehydrogenase, and probably a similar amount in malate dehydrogenase. Alkaline hydrolysis appears much less destructive of tryptophan than acid hydrolysis.

A further check on tryptophan content was obtained by gas chromatography of silylated alkaline and acid hydrolysates of malate dehydrogenase and alcohol dehydrogenase. Following alkaline hydrolysis, the areas of the silylated tryptophan peak corresponded to 1.00 to 1.12 tryptophanyl residues per molecule of malate dehydrogenase, and 11.3 residues per molecule of yeast alcohol dehydrogenase. After acid hydrolysis the corresponding values were 0.32 to 0.47 (malate dehydrogenase) and 7.0 (yeast alcohol dehydrogenase). The newer and more convenient gas chromatographic analysis thus confirmed the results with the amino acid analyzer.

**Mechanism of Tritium Transfer to Enzyme—Studies of protein-labeling by l-malate-2-H generated enzymatically from ethanol-1-H via DPN are summarized in Tables III and IV. The effect of various prior treatments of the enzyme and omission of...**
components is shown in Table III. Unlike alcohol or lactate dehydrogenases, the malate dehydrogenase was labeled at pH 8.1 as well as 9.8 (Table III, Experiments 1 and 2). Requirements for active enzyme were indicated by the effect of addition of N-bromosuccinimide, AgNO₃, or urea (Experiments 3 to 7). Malate, rather than DPNH, appeared to function in protein-labeling (Experiments 8 and 9). In this respect malate dehydrogenase behaved analogously to lactate dehydrogenase (3).

Tritium transfer to protein was independent of the method of denaturation, as shown in Table IV, again suggestive of the trapping of an equilibrium mixture of tritiated substrate, coenzyme, and enzyme.

Studies with synthetic dL-malate-2-²H confirmed the above results, as seen in Table V. In addition, the DEAE-cellulose-purified enzyme was shown to be labeled similarly to the untreated enzyme (Experiment 2), and the radiochemical purity of the malate and identity of malate as the transferring species were shown by isotope dilution (Experiment 6). Malate dehydrogenase, in a manner similar to alcohol and lactate dehydrogenase, thus becomes labeled by tritiated substrate or coenzyme when the active enzyme in equilibrium with its substrates is denatured by acid, heat, or alkali. Malate dehydrogenase is similar to lactate dehydrogenase in its nonrequirement for coenzyme, and appears to acquire tritium at near neutral pH.

The evidence for tryptophan in malate dehydrogenase presented here must be reconciled with the earlier studies (4-7) of Kaplan, Thorne, Kitto and various workers who concluded on the basis of their evidence from fluorescence measurements and lack of reactivity with Ehrlich's reagent, that tryptophan was absent in this mitochondrial malate dehydrogenase and certain other malate dehydrogenases. The absence of fluorescence typical of tryptophan might be due to the presence of a nearby polar or polarizable group that quenches fluorescence. This possibility is suggested by the work of Shifrin (22) who found that indole attached to position 3 through a a-carbon chain to a quaternary nitrogen cation was nonfluorescent. Similarly Cowgill found the fluorescence quenched in indoles attached to sulphydryl or disulfide groups (23). Quenching may also be due to a thioether group. Thus we recently found that compounds such as I exhibited about 2 to 3% of the fluorescence of compound II, which showed normal indole fluorescence.

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