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PURIFICATION AND PROPERTIES OF OSTRICH HEART MALATE DEHYDROGENASES

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SUMMARY

The supernatant form of malate dehydrogenase (L-malate:DPN oxidoreductase, EC 1.1.1.37) has been purified from the heart of a Masai ostrich *(Struthio camelus)*. The enzyme was crystallized and was found to be homogeneous on ultracentrifugal analysis, having an $s_{20,w}$ value of 3.8. The properties of this enzyme have been compared with the properties of partially purified ostrich mitochondrial malate dehydrogenase and with crystalline chicken supernatant and mitochondrial malate dehydrogenases. Both ostrich enzymes have properties very similar to their counterparts in chicken. The amino acid compositions of ostrich and chicken supernatant malate dehydrogenases are strikingly similar.

INTRODUCTION

Over the past several years the purification and properties of malate dehydrogenase (L-malate:DPN oxidoreductase, EC 1.1.1.37) from a variety of sources have been reported. The organisms studied include human[1], cow[2–7], horse[8,9], pig[8–11], rabbit[8], rat[8, 12–14], pigeon[8], *Neurospora crassa*[15], and *Bacillus subtilis*[16,17] species. In this laboratory we have also purified malate dehydrogenases from chicken, tuna, and *Escherichia coli*. The unusual availability of an ostrich heart provided an opportunity to extend our comparative studies with malate dehydrogenases and in particular made possible a comparison of the enzymes from two avian species.

MATERIALS AND METHODS

Materials

The ostrich heart (approx. 500 g) was from a Masai ostrich *(Struthio camelus)* which was obtained through the courtesy of the Bronx Zoo. Other materials were as described in the preceding paper[18].

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Methods

Except as detailed below, all methods employed were those described in the preceding paper[18]. For experiments with the crystalline ostrich supernatant malate dehydrogenase an experimentally determined extinction coefficient of $E^{1\%}_{1\text{ cm, }280\text{ m}\mu} = 12.8$ was used.

Tryptophan analyses

The tyrosine:tryptophan ratio was obtained according to the procedure of BENCZE AND SCHMID[19] and GOODWIN AND MORTON[20]. These were converted into tryptophan content by assuming the tyrosine residue value obtained by amino acid analysis was correct. In the first procedure, a tangent was drawn to the absorption peaks at 290 and 282 mμ. The resulting slope was converted into a tyrosine: tryptophan ratio with the constants given by these authors. From the same spectra, the absorbance values at 280 and 294.4 mμ, corrected for extraneous absorption, were used to determine the ratio from the formula obtained by GOODWIN AND MORTON.

Fluorescence studies

The fluorescence spectrum of ostrich supernatant malate dehydrogenase was determined on a Zeiss spectrofluorimeter Model ZFM 4c. Chicken-heart lactate dehydrogenase, tyrosine and tryptophan were used as standards. The spectra were corrected for variations in the photomultiplier efficiency and monochromater dispersion with varying wavelengths.

The formula used for the calculation of quantum yield (Q) was: $\frac{A_1}{A_2} = \frac{Q_1(FA_1)}{Q_2(FA_2)}$, where A_1 is the area under the emission curve of the protein solution from 290 to 460 mμ; A_2 is the corresponding area for the protein standard (in this case chicken-heart lactate dehydrogenase); Q is the unknown quantum yield of the protein solution; FA_1 is the fractional absorbance of the protein solution; FA_2 is the fractional absorbance of the protein standard. Fractional absorbance is defined by the formula: $1 - T/100$, where T is the per cent transmission of the solution for a pathlength of 1 cm. All absorbance measurements were done with a quartz cell of 5 cm pathlength using a Zeiss spectrophotometer Model PMQ II.

Enzyme purification

The methods used for the purification and crystallization of the ostrich supernatant malate dehydrogenase were essentially those described previously for the purification of chicken supernatant malate dehydrogenase[21], except that all procedures were scaled down in proportion to the smaller amount of starting material available. Because a number of other enzymes were being purified from a single ostrich heart it was not possible to retain more than a small sample of the ostrich mitochondrial malate dehydrogenase which was separated from the supernatant malate dehydrogenase by chromatography on carboxymethylcellulose. The ostrich mitochondrial malate dehydrogenase obtained at this stage was estimated, from specific activity, to be approx. 75% pure. It was used without further purification in the studies described below.

After three recrystallizations, the ostrich supernatant malate dehydrogenase

was stored as a crystalline suspension in $(NH_4)_2SO_4$ containing 0.1 M phosphate buffer + 0.01 M β-mercaptoethanol (pH 7.5). Approx. 30 mg of crystalline enzyme were obtained.

RESULTS AND DISCUSSION

Ultracentrifuge studies and molecular weight determinations

A sample containing 10 mg of crystalline ostrich supernatant malate dehydrogenase in 0.1 M phosphate buffer was examined in the ultracentrifuge at 59 780 rev./min. The protein sedimented as a single symmetrical peak with an $s_{20,w}$ of 3.8. This value is very similar to the sedimentation constants obtained previously for chicken[21] and pig supernatant malate dehydrogenases[22] and for mitochondrial malate dehydrogenases from chicken[21], pig[9], horse[9], and tuna[18], suggesting that all these enzymes are of comparable molecular size.

The molecular weights of the ostrich supernatant and mitochondrial malate dehydrogenases were estimated by gel-filtration on Sephadex G-100 columns calibrated for molecular weight determination with proteins of known molecular weight. Both ostrich malate dehydrogenases had identical elution volumes which corresponded to a mol. wt. of 67 000 ± 5000. Chicken, pig, and tuna supernatant and mitochondrial malate dehydrogenases examined by this procedure also had elution volumes identical to those of the ostrich malate dehydrogenases.

TABLE I

AMINO ACID COMPOSITION OF SUPERNATANT MALATE DEHYDROGENASES

All values are reported as residues per mole of enzyme (mol. wt., 70 000).

Residue	*Supernatant enzymes*			
	Ostrich	*Chicken**	*Pig***	*Beef****
Lys	65 ± 4.5†	57	57	67
His	10 ± 1.2	11	10	7
Arg	20 ± 2.2	20	19	19
Asp	66 ± 3.1	68	70	67
Thr	23 ± 3.1	30	29	24
Ser	21 ± 3.6	39	42	30
Glu	64 ± 1.4	60	59	55
Pro	27 ± 2.0	29	27	21
Gly	44 ± 2.2	59	43	42
Ala	57 ± 1.5	60	61	55
Val	62 ± 2.6	53	51	47
Met	16 ± 1.1	14	15	15
Ile	39 ± 1.8	42	40	41
Leu	61 ± 2.2	59	60	62
Tyr	15 ± 1.4	16	15	14
Phe	23 ± 0.8	24	24	19
Try	11††	12	16	12

* Data from KITTO AND KAPLAN[21].
** Data from THORNE AND COOPER[22].
*** Data from SIEGEL AND ENGLARD[4].
† Standard deviation. Triplicate analyses were made of 24- and 48-h hydrolysates.
†† See ref. 18 for details of analysis.

Amino acid composition

The amino acid composition of the ostrich supernatant malate dehydrogenase is given in Table I, together with the compositions of the supernatant malate dehydrogenases of chicken[21], pig[22], and beef[4] for comparison. The four enzymes show few significant differences in amino acid composition. The valine content of the ostrich supernatant malate dehydrogenase appears to be somewhat greater than that found in the other species, as does the glutamic acid content. The low serine value obtained with the ostrich enzyme probably represents loss of this residue during hydrolysis rather than actual difference in composition between the ostrich and the other enzymes. In contrast to mitochondrial malate dehydrogenases, which lack tryptophan[9,21], the supernatant forms of malate dehydrogenase contain this amino acid.

Starch-gel electrophoresis

The supernatant and mitochondrial forms of ostrich malate dehydrogenase can readily be separated by starch-gel electrophoresis as shown in Fig. 1. When duplicate slices of a starch-gel electrophoresis of the crystalline ostrich supernatant malate dehydrogenase were stained for protein and for enzymatic activity, no traces of protein components, other than that associated with activity, could be detected. The electrophoretic mobilities of the ostrich supernatant and mitochondrial malate dehydrogenases were found to be indistinguishable from the mobilities of the corresponding enzymes of two other flightless birds, Pertriz tinamou *(Nothura maculosa)* and Darwin's rhea *(Rhea pennata)* (Fig. 1).

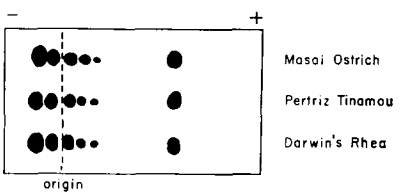

Fig. 1. A tracing of a starch-gel electrophoresis showing the electrophoretic mobility of the supernatant and mitochondrial malate dehydrogenases of ostrich *(Struthio camelus)*, tinamou *(Nothura maculosa)* and rhea *(Rhea pennata)*. Electrophoresis was carried out in phosphate–citrate buffer (pH 7.0) for 16 h at 4°.

Fluorescence and quantum yield

The fluorescence spectrum of ostrich supernatant malate dehydrogenase is presented in Fig. 2, with the spectra of chicken-heart lactate dehydrogenase and chicken mitochondrial malate dehydrogenase and tyrosine for comparison. The fluorescence maximum of the ostrich supernatant malate dehydrogenase is at 335 mμ, when the protein is excited at 280 mμ. In this respect, the ostrich supernatant enzyme is like chicken-heart lactate dehydrogenase and other tryptophan-containing proteins which characteristically show fluorescence maxima between 330 and 345 mμ (ref. 23).

The fluorescence spectrum of ostrich supernatant malate dehydrogenase was found to be virtually identical with that of chicken supernatant malate dehydrogenase, but as shown in Fig. 2, differed markedly from the spectrum of chicken

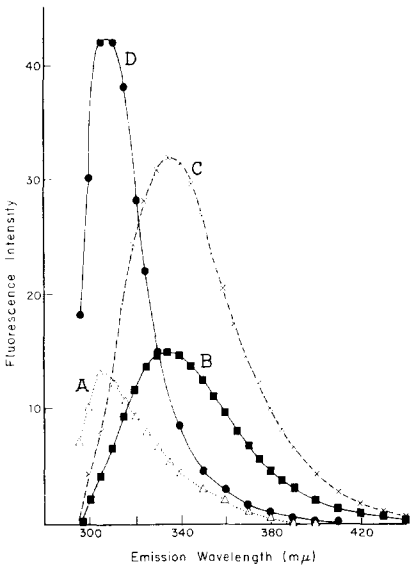

Fig. 2. Fluorescence emission spectra of solutions of tyrosine, ostrich supernatant malate dehydrogenase, chicken mitochondrial malate dehydrogenase, and chicken-heart lactate dehydrogenase, in 0.1 M phosphate buffer (pH 7.5). All measurements were made in a Zeiss spectrofluorimeter Model ZFM4c at 25°, the excitation wavelength being 280 mμ. A, chicken mitochondrial malate dehydrogenase, $A_{280\ m\mu}$, 0.1; B, ostrich supernatant malate dehydrogenase, $A_{280\ m\mu}$, 0.008; C, chicken-heart lactate dehydrogenase, $A_{280\ m\mu}$, 0.009; D, tyrosine, $A_{280\ m\mu}$, 0.02 .

mitochondrial malate dehydrogenase, which contains no tryptophan, and has a fluorescence maximum at 307 mμ.

The fluorescence quantum yield of ostrich supernatant malate dehydrogenase was calculated on the basis of the published values of 24% for chicken-heart lactate dehydrogenase[24], and 21% for tryptophan[23,25] values between 13–15% being determined for the native ostrich enzyme. Correction of the quantum yield to the absorption of only the tryptophan residues was made by assuming the tryptophan: tyrosine ratio and tyrosine contents obtained on amino acid analysis to be correct. Subtraction of that proportion of fluorescence due to tyrosine gave a quantum yield per tryptophan of 17–18%, which is considerably less than the 31% reported for chicken-heart lactate dehydrogenase[24]. Such a difference, however, is not unexpected, since TEALE[23] found the quantum yields of many different tryptophan-containing proteins to vary from 7–48%.

Immunological studies

A rabbit antibody was prepared against the crystalline ostrich supernatant malate dehydrogenase. This antiserum was shown to give a single sharp precipitin band when tested by double diffusion in agar against the crystalline supernatant malate dehydrogenase and against a crude ostrich heart extract. No cross-reaction was obtained with partially purified ostrich mitochondrial malate dehydrogenase or with crystalline chicken mitochondrial malate dehydrogenase. The anti-ostrich supernatant malate dehydrogenase serum gave a single complement fixation peak with ostrich supernatant malate dehydrogenase and an equally strong cross-reaction

TABLE II

INHIBITION OF MALATE DEHYDROGENASES BY AN ANTIBODY TO OSTRICH SUPERNATANT MALATE DEHYDROGENASE

The enzymes were incubated at 0° for 15 min with a 1:150 dilution of an antibody (Ra 480-B5) to ostrich supernatant malate dehydrogenase prior to assays for enzymatic activity.

Malate dehydrogenase	*% Inhibition*
Ostrich supernatant	40
Ostrich mitochondrial	0
Chicken supernatant	33
Chicken mitochondrial	0

with chicken supernatant malate dehydrogenase. As shown in Table II, the anti-ostrich supernatant malate dehydrogenase strongly inhibits the enzymatic activity of both ostrich and chicken supernatant malate dehydrogenases, but is without effect on the mitochondrial malate dehydrogenases of these species.

Catalytic properties

The specific activity of the crystalline ostrich supernatant malate dehydrogenase was determined using the assay conditions of THORNE AND COOPER[22]. Under these conditions the ostrich enzyme had a specific activity of 75 units/mg, which is somewhat greater than the above authors reported for pig supernatant malate dehydrogenase. With DPNH, the rate of oxaloacetate reduction was approx. 5 times as great as the optimal rate of malate oxidation.

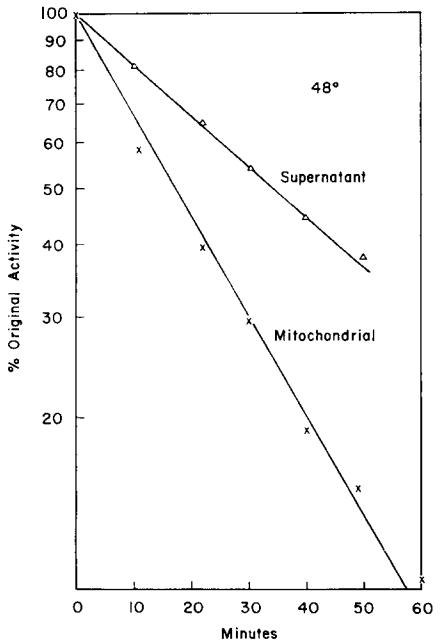

Fig. 3. Rates of thermal inactivation of ostrich supernatant and mitochondrial malate dehydrogenases at 48°.

As has been found in a number of comparisons of supernatant and mitochondrial malate dehydrogenases from different sources, the ostrich mitochondrial malate dehydrogenase was more susceptible to inhibition by high concentrations of oxaloacetate than was the supernatant malate dehydrogenase. High concentrations of malate gave more inhibition of the ostrich supernatant malate dehydrogenase than of the mitochondrial malate dehydrogenase.

Using non-inhibiting concentrations of oxaloacetate, a K_m for this substrate of $3 \cdot 10^{-5}$ was determined for crystalline ostrich supernatant malate dehydrogenase by a reciprocal plot.

The supernatant and mitochondrial ostrich enzymes have different susceptibilities to inactivation by heat, the mitochondrial enzyme being somewhat more thermolabile, as shown in Fig. 3. This differential heat stability is similar to that found for the two malate dehydrogenases from chicken[18], but is much less marked than the differences in stability of the heart and muscle forms of chicken lactate dehydrogenase[26].

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