A Reappraisal of some Structural Features of Bovine Heart Malate Dehydrogenase

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1. Malate dehydrogenase of the mitochondrial type was prepared from an acetone-prepared powder of thoroughly washed minces of whole bovine heart by previously reported methods that were modified to give higher yields of the purified enzyme. 2. Determinations of the sedimentation and diffusion coefficients showed the molecular weight of the enzyme to be approx. 63 000. The amino acid composition of the enzyme was also determined. Discrepancies between these data and similar data previously reported by Davies & Kun (1957) and Siegel & England (1962) were resolved. 3. 'Fingerprints' were made from tryptic digests of heat-denatured and of reduced and alkylated enzyme. These indicated that the enzyme is composed of a number of identical or similar sub-units.

Reports of methods for isolating malate dehydrogenase (L-malate-NAD oxidoreductase, EC 1.1.1.37) from bovine heart reveal that there are two types of this enzyme within the tissue, one type being characteristic of the cytoplasm and the other of the mitochondria (Davies & Kun, 1957; Siegel & England, 1961; England & Breiger, 1962; Siegel & England, 1962). Davies & Kun (1957), starting their preparation from an acetone-dried powder of isolated mitochondria, obtained a malate dehydrogenase that they reported to have a molecular weight of 15 000–20 000. Later preparations of the mitochondrial form of this enzyme, which were reported by Siegel & England (1961) and by Grimm & Doherty (1961), started from acetone-dried powders of washed minces of whole bovine heart: in both instances, the purified enzyme was shown to have a molecular weight of 62 000 or thereabouts. In spite of this evidence, Kun (1963) maintained that mitochondrial malate dehydrogenase has the lower molecular weight and that forms with higher molecular weights may originate in the cytoplasm.

We have sought to resolve this disagreement on the molecular weight of malate dehydrogenase by supplying independent data supporting the estimate of Siegel & England (1961) and by showing that this value is consistent with the major component of the preparation of Davies & Kun (1957).

The kinetic properties of our preparation with regard to inhibition by oxaloacetate are characteristic of the mitochondrial form of the enzyme and quite distinct from those possessed by the cytoplasmic form (Siegel & England, 1960, 1961; Cassman & England, 1966). A separate study (Heyde &

Ainsworth, 1968) contains details of a kinetic investigation of the mechanism of the reaction catalysed by our preparation.

We also report data on the amino acid composition of malate dehydrogenase and the number of peptides that are produced by tryptic digestion.

EXPERIMENTAL

Materials. The sodium salt of NAD⁺ (grade III, 98%) was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). British Drug Houses Ltd. (Poole, Dorset) supplied pyridine, glycine and sodium hydrogen malate (laboratory-reagent grade) as well as acetic acid, butan-1-ol and EDTA (analytical-reagent grade). Tris (British Drug Houses Ltd.) was recrystallized from solution in water at 60° by the addition of an equal volume of acetone. Crystalline bovine plasma albumin was the product of Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex). A di-isopropyl phosphorofluoridate-treated sample of trypsin was purchased from the Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Iodoacetic acid was obtained from Mann Research Laboratories Inc. (New York, N.Y., U.S.A.), 2-mercaptoethanol from Eastman Kodak Co. (Rochester, N.Y., U.S.A.), and *tert.*-butyl hypochlorite from the Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.).

Preparation of malate dehydrogenase. The method used was essentially that of Straub (1942), as modified by Ochoa (1955) for the preparation of pig heart malate dehydrogenase, and employed by Siegel & England (1961) to prepare bovine heart mitochondrial malate dehydrogenase. The precautions of Pfeleiderer & Hohnholz (1959) for the addition of CaCl₂ and ethanol were observed.

An exhaustively washed mince of whole bovine heart was extracted with cold acetone (–15°). The excess of acetone was removed by squeezing the mince in a gauze cloth, but the powder was not dried for we found that omission of this

step considerably increased the final yield of enzyme. The mince was then extracted with 0.1M-sodium phosphate buffer, pH 7.4 at 2°, and the extract treated by adding ice-cold 15% (w/v) CaCl_2 . The resulting calcium phosphate precipitate was centrifuged off at 0° and discarded. The supernatant was immediately neutralized by adding ice-cold 10% (w/v) $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$. The precipitate of calcium phosphate formed during neutralization was found to adsorb the enzyme; elution was effected by 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ at 2°. All the protein precipitated by increasing the $(\text{NH}_4)_2\text{SO}_4$ concentration to 70% saturation (and occasionally slightly higher) was fractionated with ethanol. A final $(\text{NH}_4)_2\text{SO}_4$ fractionation at 2° yielded most of the enzyme between 60% and 75% saturation. The controlled heating step of Siegel & England (1961) was not performed to avoid the possibility of destroying delicate tertiary structure such as might be involved in an allosteric site. The enzyme was dissolved in tris-acetate buffer, pH 7.5 (0.05M with respect to acetate), dialysed against the same buffer and then stored at 2° in concentrations of approx. 10mg. of protein/ml. All preparations used were homogeneous in the analytical ultracentrifuge, and had specific activities usually between 300 and 400 μmoles of NADH produced/min./mg. of protein under the standard assay conditions. Details of a typical preparation are recorded in Table 1.

Standard assay conditions. The assay of Siegel & England (1961) was used, with the addition of EDTA to give a final concentration of 1mM. This addition enhanced the initial reaction rate in early stages of the preparation, but later had no effect.

Protein determination. The method used was that of Gornall, Bardawill & David (1949), with crystalline bovine plasma albumin (water content 5%) as the standard.

'Fingerprinting'. (a) Heat-denaturation of enzyme. A stock solution of malate dehydrogenase was dialysed against 1mM-EDTA, pH 8.5, and then against water adjusted to pH 8.5, to remove salts. The salt-free solution was heated in a boiling-water bath for 4min. to denature the enzyme. Then 0.8M-ammonium bicarbonate buffer, pH 8.5, was added to give a final buffer concentration of 0.2M.

(b) Reduction and alkylation of enzyme. A 12mg. sample of malate dehydrogenase was reduced with 2-mercapto-ethanol and alkylated with iodoacetic acid, in a total volume of 5ml., by the method of Crestfield, Moore & Stein (1963). After the alkylation reaction had proceeded for 15min., the protein was precipitated by adding 5 vol. of acid acetone at -15° (Sela, White & Anfinsen, 1959) and washed three times with this by centrifugation. The washed precipitate

was dissolved in 0.2M-ammonium bicarbonate buffer, pH 8.5.

(c) Tryptic digestion. Malate dehydrogenase was incubated at 38° in 0.2M-ammonium bicarbonate buffer, pH 8.5, with 2% (w/w) of di-isopropyl phosphorofluoridate-treated trypsin. After 4hr. a second portion of trypsin was added, making the total concentration 4% (w/w) of the malate dehydrogenase. The reaction was allowed to proceed for a further 5hr., after which the digest was stored at -15°. Protein that remained insoluble was removed by centrifugation before subjecting the digest to chromatography and electrophoresis. The supernatant from the digest of heat-denatured protein contained 45% of the original protein, and 83% of the digest of reduced and alkylated protein was soluble.

(d) Chromatography and electrophoresis. A portion of tryptic digest of malate dehydrogenase, containing approx. 2mg. of protein, was applied to Whatman no. 3MM chromatography paper (18in. $\times$ 22in.). Descending chromatography with the butanol phase from the mixture butan-1-ol-acetic acid-water (4:1:5, by vol.) was allowed to proceed for 30hr. After the paper had been dried for 30min. at 70°, electrophoresis was performed in pyridine-acetic acid-water (1:10:289, by vol.), pH 3.7, at 2000V and 80A for 2hr., with a Miles Hivolt high-voltage electrophoresis apparatus. The paper was dried at 70° and stained, either by dipping in a 3% (w/v) solution of ninhydrin in ethanol or by spraying first with *tert.*-butyl hypochlorite in cyclohexane, aerating at room temperature and spraying finally with starch-iodide (Mazur, Ellis & Cammarata, 1962).

Amino acid analysis. A solution of malate dehydrogenase was dialysed to remove salts, as described above, and divided into portions each containing approx. 2mg. of protein. These were freeze-dried and hydrolysed in sealed tubes with 6N-HCl at 110° for various periods before analysis was carried out in an EEL automatic analyser, according to the procedure of Spackman, Stein & Moore (1958). A similar sample was oxidized with performic acid (Moore, 1963) before hydrolysis with HCl and analysis.

RESULTS

Determination of molecular weight

Sedimentation coefficients. Five samples of purified enzyme, dialysed against tris-acetate buffer (0.05M with respect to acetate, pH 7.5) and ranging in concentration from 2.4 to 14.0mg./ml., were

Table 1. Preparation of malate dehydrogenase

Fraction	Vol. (ml.)	Total activity (μmoles of NADH produced/min.)	Total protein (mg.)	Sp. activity (μmoles of NADH produced/mg.)	Recovery (%)	Purification factor
1. Extract of acetone-treated washed mince	1500	105000	24000	4.4		
2. Na_3PO_4 ppt.	1100	31800	2970	10.7	30	2.4
3. 50-70%-satd. $(\text{NH}_4)_2\text{SO}_4$ ppt.	40	26900	316	85	26	19
4. Ethanol ppt.	18	21200	162	131	20	30
5. 60-75%-satd. $(\text{NH}_4)_2\text{SO}_4$ ppt.	2.5	13600	35	388	12.8	88

Fig. 1. Schlieren patterns of malate dehydrogenase (7.7 mg./ml.) in 0.05 M-tris-acetate buffer, pH 7.5, obtained with the Spinco model E ultracentrifuge. Conditions were as follows: temperature, 7.5°; rotor speed, 59780 rev./min.; bar angle 60° for the first two exposures, 50° for the others. Photographs were taken at 16 min. intervals after reaching full speed. Migration is from right to left.

Table 2. Variation of $S_{20,w}$ with protein concentration

$S_{20,w}$ (s)	Concn. of protein (mg./ml.)
4.34	2.4
4.08	7.7
4.06	10.0
4.09	13.6
3.86	14.0

Table 3. Variation of $D_{20,w}$, determined from synthetic-boundary experiments, with protein concentration

$10^7 D_{20,w}$ (cm. ² sec. ⁻¹)	Concn. of protein (mg./ml.)
6.8	1.5
6.4	3.0
6.1	6.0
6.2	7.5
6.0	8.5

examined in the Spinco model E ultracentrifuge. The enzyme sedimented as a single component; this is shown in Fig. 1, where photographs of the sedimenting boundary obtained with a sample containing 7.7 mg./ml. are reproduced. There was a slight dependence of $S_{20,w}$ on protein concentration over the range studied (Table 2) and extrapolation of $S_{20,w}$ to infinite dilution, with a least-squares fit to the data, yielded a value of 4.44×10^{-13} sec. This agrees with the values reported by Siegel & England (1961) and by Grimm & Doherty (1961) from measurements over a narrower range of protein concentration. It is also essentially in agreement with the single value (2.1×10^{-13} sec. at 2°) reported by Davies & Kun (1957), provided that the marked correction for temperature is applied to this value (3.5×10^{-13} sec. at 20°). The slight increase in $S_{20,w}$

with decrease in protein concentration is in agreement with the findings of Grimm & Doherty (1961), but contrasts with the positive correlation recorded for the pig heart enzyme (Wolfe & Neilands, 1956).
Diffusion coefficients. (a) From synthetic-boundary experiments. Determinations of D were made on five samples of enzyme, ranging in concentration from 1.5 to 8.5 mg./ml. and dialysed as before, the synthetic-boundary cell being used in the ultracentrifuge (the centrifugal field was 3600g). D at each concentration was determined from the slope of a least-squares fit to the plot of σ^2 , i.e.:

$$\frac{1}{2\pi} \left(\frac{\text{area of peak}}{\text{maximum height}} \right)^2$$

against time (Schachman, 1955). The area of the peak was corrected throughout by the factor $(\bar{x}/x_0)^2$, where $\bar{x}$ is the distance of the peak from the centre of rotation at time t and x_0 is the corresponding distance at $t = 0$, to compensate for dilution of the protein as it moved down the broadening cell. There was an increase in $D_{20,w}$ with decrease in protein concentration (Table 3), and extrapolation to infinite dilution by means of a least-squares fit to the data yielded a value of 6.8×10^{-7} cm.²sec.⁻¹. This is slightly higher than the value of 6.45×10^{-7} cm.²sec.⁻¹ reported by Siegel & England (1961), but threefold lower than the estimate of 13.5×10^{-7} cm.²sec.⁻¹ at 2° made by Davies & Kun (1957) (23.9×10^{-7} cm.²sec.⁻¹ at 20°).
(b) From sedimentation-velocity experiments. Values of D were also calculated from the broadening of peaks in photographs of schlieren patterns observed during sedimentation experiments. The photographs in Fig. 1 yielded a value for $D_{20,w}$ of 6.3×10^{-7} cm.²sec.⁻¹, corrected to 6.5×10^{-7} cm.²sec.⁻¹ by allowing for the dependence of the sedimentation coefficient on protein concentration (Baldwin, 1957) as well as for dilution of the protein as it moves down a broadening cell. These,

Table 4. *Amino acid composition of malate dehydrogenase*

The content of each amino acid is expressed as residues/mol. of enzyme, based on an arginine content of 15.0 residues/mol. Values in the last columns are means of those in the previous columns, except where a closer approximation to the true content is given by either extrapolation to zero time of hydrolysis by means of a least squares fit to the data (*), or the final estimate after the longest period of hydrolysis (**). The analyses in the first column were made on a performic acid-oxidized sample of enzyme.

Time of hydrolysis (hr.) ...	Composition (residues/mol.)					Mean, extrapolated or final	Rounded
	20.5	22	44	68			
Amino acid							
Aspartic acid	45.0	44.4	45.1	45.3		45.0	45
Threonine	36.3	36.1	34.6	32.1		38.0*	38
Serine	26.5	28.8	21.8	19.3		31.1*	31
Glutamic acid	45.0	48.0	50.0	49.1		48.0	48
Proline	—	36.8	41.5	41.1		39.8	40
Glycine	49.0	49.9	50.0	49.8		49.6	50
Alanine	57.4	60.4	62.0	60.0		60.0	60
Valine	50.6	47.5	51.0	53.3		50.6	51
Methionine	10.8	9.9	11.0	12.3		12.3**	12
Isoleucine	37.0	36.1	40.8	42.3		42.3**	42
Leucine	48.5	48.9	50.1	51.9		51.9**	52
Tyrosine	—	9.7	7.4	6.5		11.0*	11
Phenylalanine	—	21.9	22.8	21.6		22.1	22
Cysteic acid	16.3	—	—	—		16.3	16
Lysine	49.6	45.3	44.9	47.9		46.9	47
Histidine	—	10.4	9.6	9.8		10.0	10
Arginine	15.0	15.0	15.0	15.0		15.0	15

and other values, are in general agreement with the $D_{20,w}$ values determined from synthetic-boundary experiments (Table 3).

The value of D employed by Davies & Kun (1957) in calculating the molecular weight of malate dehydrogenase was obtained in free-diffusion experiments. The presence of a small impurity would be expected to affect this value. An independent determination of the diffusion coefficient of the main component of this preparation can be made from the broadening of the major peak in photographs of schlieren patterns observed during a sedimentation experiment. The value obtained from the final two exposures obtained by Davies & Kun (1957) was $5.7 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ at 12.9° . This value corresponds to $6.9 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ at 20° , and thus is very similar to $D_{20,w}$ values reported for bovine heart mitochondrial malate dehydrogenase by all other groups.

Partial specific volume. The partial specific volume, $\bar{v}$, was calculated to be 0.75 ml./g. by the method of Cohn & Edsall (1943), from the rounded values for the amino acid composition given in Table 4. This is to be compared with the value of 0.74 ml./g. assumed by Siegel & England (1961) and the value of 0.75 ml./g. assumed by Davies & Kun (1957) and Grimm & Doherty (1961).

Molecular weight. From the values $S_{20,w}$ $4.44 \times 10^{-13} \text{ sec.}$, $D_{20,w}$ $6.80 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ and $\bar{v}$ 0.75 ml./g., a molecular weight of 63300 may be calculated for our preparation of malate dehydrogenase. This may be compared with values of 62000 calculated by Siegel & England (1961) and 65000 calculated by Grimm & Doherty (1961).

Amino acid composition

Details of analyses performed after various periods of hydrolysis of the protein are given in Table 4. When the composition is based on an arginine content of 15.0 residues/mol. the molecular weight that can be calculated, neglecting the presence of tryptophan, is 62600. This is consistent with the molecular weight determined by ultracentrifugal methods, and may be compared with values of 66700, 58500 or 50300 if the arginine content is taken as 16.0, 14.0 or 12.0 residues/mol. respectively.

There is a general similarity between these results and those reported by Siegel & England (1962), though the total number of residues/mol. appears greater in the present work because allowance has been made for the elimination of a molecule of water for each peptide bond formed. The present analysis indicates a lower content of aspartic acid, glutamic

Fig. 2. 'Fingerprint' from a tryptic digest of heat-denatured malate dehydrogenase. Chromatography was in butan-1-ol-acetic acid-water to right, followed by electrophoresis in pyridine-acetic acid-water in the vertical direction. Ninhydrin staining was used.

acid, glycine and phenylalanine by comparison with the arginine content.

An attempt to determine the tryptophan content of the enzyme by the method of Spies & Chambers (1949) as modified by Burton & Waley (1966) resulted in a value of 0.46 residues/mol. The minimum molecular weight predicted on this basis was 138 000, which does not agree with the physicochemical evidence. This content represents about half that reported by Siegel & England (1962), who used the spectrum of the enzyme in alkaline solution to estimate the amount of tryptophan present. Neither method is above criticism, and the precise tryptophan content of malate dehydrogenase may be higher than both these estimates, in view of the 'fingerprint' evidence.

'Fingerprints'. The number of peptides expected from a tryptic digest of malate dehydrogenase on the basis of the amino acid composition in Table 4 is 63 (lysine plus arginine plus one) if the enzyme is a monomer. On the 'fingerprint' (Fig. 2) 13 spots appeared reasonably definite, with three to five more doubtful ones. It would not appear likely that disulphide bridges are the cause of the low number of spots, since the content of thiol groups appears very similar to that of total cysteic acid determined

by performic acid oxidation (Siegel & England, 1962). The number of residues of cysteic acid is low and at most the number of detectable peptides could be decreased by only eight owing to the presence of disulphide bonds. Moreover, an identically produced 'fingerprint' from a digest of reduced and alkylated enzyme did not demonstrate the presence of significantly more peptides, though the spots were more diffuse. To check that very large peptides that stain poorly with ninhydrin had not been overlooked, a 'fingerprint' from a sample of heat-denatured protein was stained with *tert.*-butyl hypochlorite (Mazur *et al.* 1962). The pattern of spots so obtained was very similar to the other 'fingerprints' with no indication of a major extra peptide.

It thus appears likely that the malate dehydrogenase molecule consists of a number of identical sub-units.

DISCUSSION

The physicochemical measurements made on the present preparation of malate dehydrogenase indicate that disagreement between earlier workers over the molecular weights of enzyme prepared in different ways can be resolved. In consequence, we may set aside the suggestion made by Kun (1963) that different catalytically active fragments may be prepared from the one enzyme protein.

The amino acid analysis of the present preparation shows minor differences from that of Siegel & England (1962), though it seems clear that the same molecular species is isolated by the two slightly differing preparative methods. Evidence from 'fingerprints' indicates that the enzyme is not a monomer, and that the number of sub-units present is three to four/mol. The amino acid composition of the enzyme, based on the molecular weight of 63 000 determined physicochemically, does not show an exact correlation with either proposition. However, apart from the tryptophan content, which is in doubt, a change of 1 residue/mol. for certain of the amino acids present in smaller amounts (tyrosine, cysteic acid, histidine or arginine) would be sufficient to give a reasonable fit to either a three-sub-unit or a four-sub-unit molecule.

The finding of a sub-unit structure for bovine heart malate dehydrogenase shows some similarity to evidence (Devenyi, Rogers & Wolfe, 1966) for the presence of two sub-units in pig heart malate dehydrogenase.

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