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Lactic Dehydrogenase and Cytochrome b_2 of Baker's Yeast

THE AMINO ACID COMPOSITION OF THE CRYSTALLINE ENZYME

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Appleby & Morton (1954, 1959*a, b*, 1960) have described the purification and crystallization of yeast lactic dehydrogenase, and the enzymic, chemical and physical properties of the crystalline chromoprotein, which was shown to be identical with cytochrome b_2 . This paper describes the amino acid composition as determined qualitatively by paper chromatography and quantitatively with ion-exchange-resin columns. An account of this work was given to the Australian Biochemical Society in August, 1957 (Simmonds, Appleby & Morton, 1957*a, b*; Morton, 1958).

MATERIALS

Yeast lactic dehydrogenase (cytochrome b_2). The crystalline cytochrome was prepared as described by Appleby & Morton (1959*a*). Unless otherwise stated, the material was

recrystallized three times from a solution of the combined first crystals from a number of preparations.

Gelatin. The tyrosine-free protein was a gift from Davis Gelatine Co. Ltd., Sydney, N.S.W.

β -Lactoglobulin. Three-times crystallized material was a gift from Dr F. J. R. Hird.

Organic solvents. These were purified for chromatography by distillation at reduced pressure.

EXPERIMENTAL AND RESULTS

Amino acid composition by paper chromatography

Approx. 3 mg. of once-crystallized enzyme was hydrolysed with 0.1 ml. of 5*N*-HCl in a sealed ampoule at 105° for 20 hr. After removal of HCl, the residue was dissolved in about 70 μ l. of propan-2-ol (acidified with HCl). Suitable portions (4–10 μ l.) were applied to the appropriate grade of Whatman chromatography paper. The amino acids were separated by two-dimensional chromatography as described by Dent (1947). The ninhydrin-reactive compounds of the enzyme hydrolysate were identified by comparison with similar chromatograms of a reference mixture of known

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amino acids. The results were confirmed with two-dimensional chromatography as described by Boissonnas (1950). The amino acids identified are shown in Table 1.

Attempts to detect tyrosine and histidine with the Pauly reagent (Dent, 1947) on papers developed in collidine-lutidine solvent (Dent, 1947) were unsuccessful. Because of the intense absorption due to nucleotides associated with the crystalline cytochrome (see Appleby & Morton, 1959*b*, 1960), the spectrophotometric method of Beavan & Holiday (1952) for estimation of tyrosine and tryptophan could not be used. Tyrosine was, however, detected by the following procedure. Approx. 2.5 mg. of twice-recrystallized cytochrome b_2 was hydrolysed with 5*N*-HCl as already described, the acid was removed, the residue was dissolved in water and the whole solution applied as a spot on Whatman no. 4 chromatographic paper. Reference spots of tyrosine and of hydrolysates of β -lactoglobulin and of gelatin were also applied. The chromatogram was developed in butanol-formic acid-water according to Acher & Crocker (1952). The tyrosine was detected by the reaction with α -nitroso- β -naphthol (see Acher & Crocker, 1952). No tyrosine was detected in the gelatin.

Tryptophan. Tryptophan was estimated by the method of Spies & Chambers (1949). β -Lactoglobulin, which contains 2.5% of tryptophan (Spies & Chambers, 1949), was used as a standard. Portions of β -lactoglobulin (1–4 mg.) and of cytochrome b_2 (1.5 mg.) were dissolved in 5.8 ml. of 19*N*-H₂SO₄ at room temperature (approx. 20°). After 20 min., approx. 18 mg. of *p*-dimethylaminobenzaldehyde was added to each tube and, after about 12 hr. at room temperature, 0.2 ml. of aq.

0.014% NaNO₂. After an additional 30 min. at room temperature, *E* at 590 m μ was determined with a Beckman spectrophotometer (model DU). It was found that the addition of haemin and of riboflavin phosphate to β -lactoglobulin before treatment caused no interference with the estimation of tryptophan. Hence it was concluded that these prosthetic groups (Appleby & Morton, 1954; 1959*b*) did not interfere with the estimation of tryptophan (0.82%; see Table 2) in cytochrome b_2 . It is not known whether the deoxyribose polynucleotide component (Morton, 1958; Appleby & Morton, 1960) caused any interference in this estimation.

Amino acid analysis by resin-column chromatography

The cytochrome b_2 was weighed into tubes with B19 standard ground-glass sockets, 0.5 ml. of constant-boiling HCl was added to each sample, a reflux condenser was fitted and hydrolysis was carried out on an oil bath at 110°. Duplicate samples of approx. 2 mg. each were used for the acidic and neutral amino acids, and of approx. 3.5 mg. each for the basic amino acids. One sample was hydrolysed for 20 hr. and the other for 70 hr. The hydrolysates were dried *in vacuo* over P₂O₅ and KOH at room temperature for about 3 days to remove HCl, and the residues were then dissolved in a minimum of water and transferred quantitatively to the tops of columns of Dowex-50 resin used for analysis.

The amino acids were separated by ion-exchange chromatography (Moore & Stein, 1951) and were estimated by the colorimetric procedure with ninhydrin as described by Moore & Stein (1948). The apparatus used for collecting and dispensing

Table 1. *Amino acids identified in a hydrolysate of cytochrome b_2 by separation by paper chromatography*

Once-crystallized cytochrome b_2 (3 mg.) was hydrolysed with 5*N*-HCl at 105° for 20 hr. in a sealed ampoule. After drying, the residue was dissolved in 70 μ l. of propan-2-ol, acidified with HCl and 4–10 μ l. portions were applied to filter paper. Chromatographic procedures were carried out according to the authors quoted and as described in the text. For definition of the abbreviations used for amino acids see *Biochem. J.* (1953), 55, 5.

Reference	Solvent systems used to develop chromatogram		Amino acids identified
	First system	Second system	
Dent (1947)	Water-saturated phenol	Collidine-lutidine-water	Intense spots: Ala, Asp, Glu, Gly, leucine group,* Val Weaker spots: Arg, Cys,† Lys, Ser, Thr
Boissonnas (1950)	(a) <i>tert</i> .-butyl alcohol butan-2-one	<i>tert</i> .-butyl alcohol methanol-water	Ala, Asp, Gly and Ser,‡ leucine group,* Thr, Val and two spots unidentified
	(b) Phenol-water	Propan-2-ol-water	Ala, Arg, Asp, Cys,† leucine group,* Lys
Acher & Crocker (1952)	Butan-1-ol-formic acid-water	—	Tyr

* Leucine, isoleucine and norleucine are not separated by these procedures.

† Cystine was detected as cysteic acid.

‡ Glycine and serine are not separated in this procedure.

the portions of effluent from the columns has been described by Simmonds (1954) and by Simmonds & Wood (1954). Because of the overlap between glutamic acid and proline, and the low colour yield of proline when treated with ninhydrin at pH 5.0, the effluent fractions were divided into two groups at the period when these acids were expected. One group of alternate tubes from the column was treated with ninhydrin at pH 5.0 for estimation of glutamic acid (Moore & Stein, 1948) and the second group was treated with the ninhydrin reagent of Chinard (1952) for estimation of proline.

All of the commonly occurring amino acids of proteins except tryptophan and cystine are determined by these procedures. Tryptophan was therefore estimated separately as already described. As shown in Table 1, cystine was identified in the enzyme hydrolysate by paper chromatography, but methionine was not detected. Neither methionine nor any of its oxidation products was detected in the effluents from the ion-exchange column. Hence it has been assumed that all of the sulphur (0.74%; Appleby & Morton, 1960) of the cytochrome occurs as cysteine and cystine, and the amount (expressed as $\frac{1}{2}$ -cystine) was calculated accordingly. Table 2 shows the results for the 20 and 70 hr. hydrolysates, expressed as percentage (by wt.) of the dried material.

For amino acids other than serine there appeared to be little loss during hydrolysis, and the mean of

the results for both hydrolysis periods was used as the best estimate (Table 2). Since the decomposition of serine during hydrolysis of proteins with HCl is well established (Rees, 1946; Hirs, 1954; Smith & Stockell, 1954; Smith, Stockell & Kimmel, 1954), the results for serine were linearly extrapolated to zero time to obtain the best estimate, as given in Table 2. Glutamic acid undergoes decomposition to pyrrolidonecarboxylic acid during chromatography on the resin columns and accordingly the correction factor (1.03) of Moore & Stein (1951) was used to obtain the mean value for this amino acid (Table 2).

DISCUSSION

The qualitative (Table 1) and quantitative (Table 2) analyses are in substantial agreement. It is probable that the two unidentified amino acids detected on the chromatogram according to Boissonnas (1950) were histidine and phenylalanine, which were identified by resin-column chromatography (cf. Tables 1 and 2).

Thus methionine is either absent or present in very small amount, and it has been assumed that all of the sulphur of cytochrome b_2 occurs as cystine and cysteine. If present to the extent of 0.2–0.3%, methionine would have been detected by both column and paper chromatography. The loss of lactic-dehydrogenase activity caused by

Table 2. *Amino acid analysis of crystalline cytochrome b_2 by chromatography on ion-exchange resin*

Four-times crystallized enzyme was hydrolysed in constant-boiling HCl at 110° for 20 and 70 hr. as indicated. Amino acids of the hydrolysates were separated on a column of Dowex-50 resin and estimated as described in the text, except that cystine was estimated from the total sulphur content of the enzyme and tryptophan was determined according to Spies & Chambers (1949). Results are expressed as percentage (by wt.) of the dried material.

	Amino acid content (%)			S.D.
	20 hr. hydrolysate	70 hr. hydrolysate	Mean	
Alanine	4.42, 4.51	4.36, 4.33	4.40	0.08
Amide	1.59	1.66	1.62	—
Arginine	4.68	4.48	4.58	—
Aspartic acid	9.90, 10.11	9.71, 9.84	9.89	0.17
$\frac{1}{2}$ -Cystine	—	—	2.77	—
Glutamic acid	10.62, 11.28	10.68, 10.89	10.87*	0.30
Glycine	3.54, 4.01	3.80, 4.15	3.88	0.23
Histidine	1.05	1.35	1.20	—
Isoleucine	4.86	4.49, 4.31	4.55	0.28
Leucine	9.03	8.77, 9.32	9.04	0.26
Lysine	8.46	8.06	8.26	—
Methionine	—	—	<0.3†	—
Phenylalanine	2.98, 2.65	2.73, 3.20	2.89	0.24
Proline	4.72, 5.00	4.48	4.73	0.26
Serine	3.42, 3.29	2.62, 3.34	3.51‡	—
Threonine	3.28, 3.25	3.20, 3.40	3.28	0.08
Tryptophan	—	—	0.82	—
Tyrosine	3.17, 2.92	2.87, 3.62	3.14	0.33
Valine	6.34	6.10, 6.03	6.16	0.16

* Values are corrected for formation of pyrrolidonecarboxylic acid.

† Not detected in column effluent.

‡ Value was obtained by extrapolation of results to zero time of hydrolysis.

oxidizing agents (Appleby & Morton, 1959*b*) and the inhibition by *p*-chloromercuribenzoate (Appleby & Morton, 1954, 1959*b*) indicate that cysteine is present.

Table 3 shows the amino acid nitrogen as a percentage of the total nitrogen, and the residue weight of each amino acid as a percentage of the dry weight of the enzyme. Crystalline cytochrome *b*₂ contains 15.2 ± 0.4 % of nitrogen and approx. 14.2 % of amino acid nitrogen (Appleby & Morton, 1960). The amino acids (Table 3) represent 84 % of the total nitrogen and approx. 91 % of the amino acid nitrogen. However, only 74 % of the total weight is accounted for by amino acids (Table 3). Crystalline cytochrome *b*₂ contains approx. 6 % of a deoxyribose polynucleotide, approx. 0.8 % of protohaem and approx. 0.9 % of riboflavin phosphate (Appleby & Morton, 1954, 1959*b*, 1960). Thus these results account for about 81 % of the dry weight of the enzyme. The cause of the discrepancy between the recoveries, based on nitrogen and dry-weight estimations, is not known.

Table 3 shows the number of residues of each amino acid/10⁴ g. of protein and the number of residues/mole of enzyme-bound haem. Crystalline cytochrome *b*₂ has a weight of about 80 000 g./mole

Table 3. *Amino acid composition of crystalline cytochrome b₂ (yeast lactic dehydrogenase)*

Values are derived from the results given in Table 2.

	Amino acid N as % of total N	Residue wt. as % of dry wt.	Residues/10 ⁴ g. of enzyme	Residues/mole of haem
Alanine	4.55	3.51	4.94	39.5
Amide	9.33	1.62	10.12	81.0
Arginine	9.70	4.11	2.63	21.0
Aspartic acid	6.85	8.55	7.44	59.5
$\frac{1}{2}$ -Cystine	2.12	2.35	2.31	18.5
Glutamic acid	6.81	9.54	7.39	59.1
Glycine	4.77	2.95	5.17	41.4
Histidine	2.14	1.06	0.77	6.2
Isoleucine	3.20	3.92	3.47	27.8
Leucine	6.35	7.80	6.90	55.2
Lysine	10.42	7.24	5.66	45.7
Methionine*	—	—	0	0
Phenylalanine	1.61	2.57	1.75	14.0
Proline	3.79	3.99	4.11	32.9
Serine	3.08	2.91	3.31	26.5
Threonine	2.52	2.78	2.76	22.1
Tryptophan	0.79	0.80	0.43	3.4
Tyrosine	1.60	2.83	1.73	13.8
Valine	4.85	5.21	5.26	42.1

* Not detected either in column effluent or by paper chromatography (cf. Table 1 and text).

Table 4. *Comparative amino acid compositions of cytochrome b₂ and some other proteins*

Amino acid composition is expressed as g. of amino acid/100 g. of protein. Values for cytochrome *b*₂ have been calculated for the flavohaemoprotein, free of polynucleotide (cf. Appleby & Morton, 1960).

Enzyme	...	...	Yeast alcohol dehydrogenase	Pig-heart diaphorase	Yeast cytochrome <i>b</i> ₂ (lactic dehydrogenase)	Yeast cytochrome <i>c</i>
Type of protein	...	...	Diphosphopyridine nucleotide-linked protein	Flavoprotein	Flavohaemoprotein	Haemoprotein
Reference	...	...	Lange (1956)	Rees & Savage (1958)	This paper	Nunnikhoven (1958)
Alanine			6.6	6.9	4.6	4.5
Amide			—	1.5	1.7	1.6
Arginine			4.9	4.5	4.8	3.7
Aspartic acid			8.6	10.0	10.4	11.0
$\frac{1}{2}$ -Cystine			4.4	—	2.9	1.8
Glutamic acid			6.6	12.3	11.5	10.0
Glycine			5.9	6.4	4.1	5.8
Histidine			2.5	3.3	1.3	4.0
Isoleucine			7.0	6.5	4.8	3.8
Leucine			7.1	8.0	9.5	6.8
Lysine			7.8	9.7	8.7	16.6
Methionine			5.1	2.0	<0.3	2.1
Phenylalanine			5.7	4.4	3.1	4.4
Proline			4.8	4.6	4.9	3.6
Serine			5.8	4.3	3.7	3.3
Threonine			6.6	5.3	3.5	7.2
Tryptophan			1.7	—	0.9	1.6
Tyrosine			6.0	2.9	3.3	5.7
Valine			6.9	7.0	6.5	2.6
Mol. wt. (g.)			150 000*	81 000†	75 000‡	13 200§

* Hayes & Velick (1954). † Savage (1957). ‡ Minimum weight (g.)/mole.
§ Based on dry weight; 11 800 g./mole based on total protein (Nunnikhoven, 1958).

of haem (Appleby & Morton, 1959*b*). Table 3 shows that alanine, aspartic acid, $\frac{1}{2}$ -cystine and serine would occur with exactly 0.5 of a residue/mole of haem, but no amino acids would occur with 0.5 of a residue/mole if there are two haem groups/mole of enzyme, as seems likely (Appleby & Morton, 1960).

As yet, cytochrome *c* (de Barbieri & Scevola, 1955; Ehrenberg & Theorell, 1955; Nunnikhoven, 1958; Leaf, Gillies & Pirie, 1958) is the only cytochrome other than cytochrome *b₂* for which the amino acid composition has been determined (see Morton, 1958). Table 4 shows the amino acid composition of cytochrome *b₂* (yeast lactic dehydrogenase) expressed as g. of amino acid/100 g. of polynucleotide-free enzyme, on which basis this flavohaemoprotein may be directly compared with yeast alcohol dehydrogenase, yeast cytochrome *c* and pig-heart diaphorase (Table 4). These represent a typical diphosphopyridine nucleotide-linked dehydrogenase, a haemoprotein and a flavoprotein respectively. All are directly concerned in hydrogen (or electron) transport. Table 4 shows that the flavohaemoprotein (cytochrome *b₂*) shows a general similarity in composition to each of the other proteins and especially to diaphorase, the only flavoprotein for which the amino acid composition is available. The comparative amino acid composition of cytochrome *b₂* and cytochrome *c* has been discussed elsewhere by Morton (1958). Whereas the amino acid compositions of the proteins shown in Table 4 are rather similar, they differ from the compositions of other enzymes such as catalase, pepsin and α -amylase (see Dixon & Webb, 1958). It is possible therefore that certain features of the amino acid patterns are related to their enzymic functions.

Cytochrome *b₂* thus shows no unique features of amino acid composition, except for the apparent absence (less than 0.3%) of methionine. Most proteins and almost all enzymes so far studied contain between 0.5 and 5.0% of methionine. The apparent absence of methionine in cytochrome *b₂* supports the conclusion based on other studies (Appleby & Morton, 1954, 1959*a, b*, 1960) that the crystalline material contains only one protein.

SUMMARY

1. Crystalline cytochrome *b₂* was hydrolysed with 5*N*-hydrochloric acid at 105° for 20 hr. and the liberated amino acids were separated by paper chromatography according to Dent (1947) and Boissonnas (1950). Most of the commonly occurring amino acids, except methionine, were detected.

2. The amino acids in hydrolysates obtained after 20 and 70 hr. at 110° with constant-boiling hydrogen chloride solution were separated on a

column of Dowex-50 according to Moore & Stein (1951) and estimated by the colour developed with ninhydrin. Methionine was not detected.

3. Comparison of the amino acid composition of cytochrome *b₂*, yeast cytochrome *c*, yeast alcohol dehydrogenase and pig-heart diaphorase shows considerable similarity between these several proteins.

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