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THE ISOLATION AND CHARACTERIZATION OF THE MULTIPLE FORMS OF HUMAN SKELETAL MUSCLE TRIOSEPHOSPHATE ISOMERASE

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Summary

1. Human skeletal muscle triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) was isolated and resolved by DEAE-cellulose chromatography into three major forms, A, B, and C, which comprise 97% of the total activity. The relative distribution was 25, 46 and 29% respectively.

2. The A and C forms are homodimers, $\alpha\alpha$ and $\beta\beta$, and form B is the heterodimer, $\alpha\beta$. Reassociation studies from guanidinium chloride have indicated that A, B, and C are not conformers. Although these studies revealed the existence of two different chains, the amino acid analysis showed no significant variance. Since no differences were observed in Ouchterlony and Mancini tests or in immunotitration, the three forms are assumed to be immunologically identical.

3. The three forms have the same specific activity, Michaelis constants, pH optimum, activation energy, inhibition by metabolites and heat stability. Only with increasing ionic strength did the V and K_m values differ.

4. The two polypeptide chains (α and β) appear to be identical (amino acid composition, molecular weight and antigenity), and since the electrophoretic banding pattern changed with cell aging, it is concluded that the multiple forms of trisephosphate isomerase are the consequence of minor post-synthetic alteration(s) of form A.

Introduction

Multiple forms of the glycolytic enzyme, triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1), have been

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documented in several tissues of a large number of species including man [1—12]. Extensive research has, however, not led to uniform agreement as to the number of triosephosphate isomerase forms and to their relative distribution in human tissues.

For human erythrocytes, at least three principal forms have been found [6] and two additional minor forms were observed by Peters et al. [11]. In skeletal muscle extracts, Snapka et al. [12] discovered three forms, using starch gel electrophoresis, whereas eight forms were observed by Peters et al. [11], using the same system. The other tissues contain between one [13,14] and six forms [11].

The present paper describes the resolution of human skeletal muscle enzyme into three forms, comprising about 97% of the enzyme protein. It should be noted that the kinetic and chemical properties of these forms are essentially different from those of the erythrocyte enzyme isolated by isoelectrofocusing [10].

The reason for the presence of multiple forms of the triosephosphate isomerase also remains controversial. Sawyer et al. [10] assume a genetic origin from two cistrons (based on the strong differences in their amino acid analyses between the three forms), whereas Turner et al. [15] and Peters et al. [11] have proposed an epigenetic generation for the multiple forms. Our results support the latter hypothesis.

Methods

All procedures, unless otherwise specified, were carried out at 0—4 °C.

Enzyme assay. Enzyme activity was measured spectrophotometrically at 365 nm and at 25 °C using DL-glyceraldehyde 3-phosphate as substrate [16]. One unit was defined as 1 μmol NADH consumed/min, with a molar absorption coefficient for NADH of $3.4 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$. To prevent inactivation, the enzyme was diluted in 100 mM triethanolamine-HCl buffer (pH 7.5) containing 0.1% bovine serum albumin and 2 mM dithioerythritol.

Protein determination. The protein concentration of the purified native enzyme was determined spectrophotometrically at 280 nm with an $\epsilon^{1\%}_{1\,cm} = 12.64$. The absorption coefficient was ascertained by four independent weight measurements.

In the crude enzyme fractions, protein was assayed by a modified biuret method [17] with 1/10 of the given volumes and a color-protein converting factor of 33, measured with the purified enzyme.

Electrophoresis. Cellogel gel electrophoresis and activity staining were carried out as previously described [18]. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according to the method of Laemmli [19]. The gels were stained for protein overnight with 0.025% Coomassie brilliant blue dissolved in $CH_3OH/CH_3COOH/H_2O$ (10 : 2 : 10, v/v/v) and destained using the same solvent.

Antibody titration. Chicken antiserum I against the unresolved human muscle triosephosphate isomerase was collected as described elsewhere [18]. Antiserum II against the purified A form was obtained by the same immunization procedure.

Antigenic identity between the three forms was checked with both antisera I and II by the double immunodiffusion (Ouchterlony) and single radial immunodiffusion (Mancini). Thiol groups were completely reduced prior to the immunotitration.

Thiol groups. For the titration of the sulfhydryl groups, the three purified forms were reduced by incubation for 20 h with 2 mM dithioerythritol in degassed 500 mM Tris-HCl (pH 9.0) containing 10 mM disodium EDTA, in a closed vessel. This was followed by exhaustive dialysis against degassed 100 mM Tris-HCl (pH 8.0) without dithioerythritol. Determination of thiol groups was carried out by spectrophotometric titration with 5,5'-dithiobis-(2-nitrobenzoic acid) [20].

Amino acid analysis. The enzymes were hydrolyzed in 5.7 N HCl at 105°C for periods of 24, 48, 72, 96, and 120 h. Analyses were conducted with a Durrum D-500 Autoanalyzer. Threonine and serine values were extrapolated to zero time of hydrolysis. For valine and isoleucine, the values from the 120-h hydrolysis were taken. Tryptophan was determined spectrophotometrically by the method of Edelhoch [21].

Purification of human skeletal muscle triosephosphate isomerase. Human skeletal muscle was removed from the cooled body 6 h after death. There was no difference in the triosephosphate isomerase isolated from the muscle immediately or after the tissue was frozen for several months. Also, the donor age (28, 37, 55, and 72 years) had neither an effect on the banding pattern of the multiple forms nor on the specific activity.

1 kg ground muscle tissue was extracted for 12 h with a threefold volume of 0.1 M triethanolamine-HCl, 2 mM EDTA (pH 8.0). The crude extract was centrifuged at 13 500 $\times g$ for 20 min and fractionated by the $(NH_4)_2SO_4$ technique [17]. Fraction IV (2.6—3.2 M $(NH_4)_2SO_4$) was suspended in 100 mM sodium phosphate buffer (pH 6.0). The dialyzed protein solution was added to a CM-Sephadex C-50 column (50 × 4.2 cm) previously equilibrated with the same buffer. The enzyme was eluted with the void volume (80% yield). Following extensive dialysis against 30 mM triethanolamine-HCl buffer (pH 8.0), the enzyme was added to a DEAE-Sephadex A-50 column (110 × 2.9 cm). Triosephosphate isomerase was eluted with 8 mM α-glycerolphosphate. (In some preparations, a linear NaCl-gradient was applied yielding a more impure triosephosphate isomerase). The effluent fractions containing the triosephosphate isomerase were pooled (54% yield) and dialyzed for 12 h against 3.5 M $(NH_4)_2SO_4$ in 100 mM Tris-HCl buffer (pH 8.0). The precipitated protein was removed by centrifugation, dissolved in a minimal volume of 100 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer. Following dialysis the protein solution was poured on a Sephadex G-75 Superfine column (98 × 4 cm) and eluted with column buffer. The effluent fractions containing the triosephosphate isomerase were pooled and dialyzed against 3.5 M $(NH_4)_2SO_4$ (50%). A summary of the steps in a typical purification is listed in Table I.

The rapid inactivation of triosephosphate isomerase in stored muscle reported by Dabrowska et al. [22] could not be confirmed. In muscle extracted 3 h after death, they found 85 U/mg protein, but in a muscle extract made 7 h after death, the activity decreased to 3.7 U/mg (4% of the former). In contrast to these results, we measured an activity of 140 U/mg protein in an extract made 6 h after death.

TABLE I

PURIFICATION OF TRIOSEPHOSPHATE ISOMERASE FROM HUMAN SKELETAL MUSCLE

Step	Specific activity (U/mg protein)	Recovery (%)
Crude homogenate	140	100
2.6—3.2 M $(NH_4)_2$ SO_4	590	86
CM-Sephadex chromatography	850	80
DEAE-Sephadex chromatography (elution with 8 mM α-glycerolphosphate)	5290	54
Sephadex G-75 chromatography	8700	47

Isolation of the triosephosphate isomerase subforms. The purified muscle triosephosphate isomerase was dialyzed against 15 mM triethanolamine-HCl buffer (pH 8.3) and was poured onto the DEAE-cellulose (Servacel DEAE-52) column (160 × 4.2 cm) previously equilibrated with the dialysis buffer. A linear NaCl-gradient (0—60 mM) in 15 mM triethanolamine-HCl (pH 8.3) extended over 10 l was run through the column. Effluent fractions, were pooled as shown in Fig. 1, concentrated by dialysis against 30% polyethylene glycol (M_r 20 000) to 0.02 of the original volume and then dialyzed against 3.4 M $(NH_4)_2SO_4$ in 100 mM Tris-HCl (pH 8.0).

Reversible dissociation. The three separated forms were dissolved (400 U/ml) in 15 mM Tris-citrate, 1 mM EDTA (pH 8.1). Using 10 N HCl, the pH was adjusted while stirring to pH 3.5, then immediately increased to pH 9.5 with 8 N NaOH, and finally lowered again to pH 8.1 with HCl. In a second experiment, the three forms (5 mg/ml) were incubated in 6 M guanidine-HCl containing 2 mM EDTA, 2 mM dithioerythritol and 10 mM Tris-HCl (pH 6.0) for 1 h at room temperature. Following incubation, the enzyme solution was diluted (1 : 700) in electrophoresis buffer.

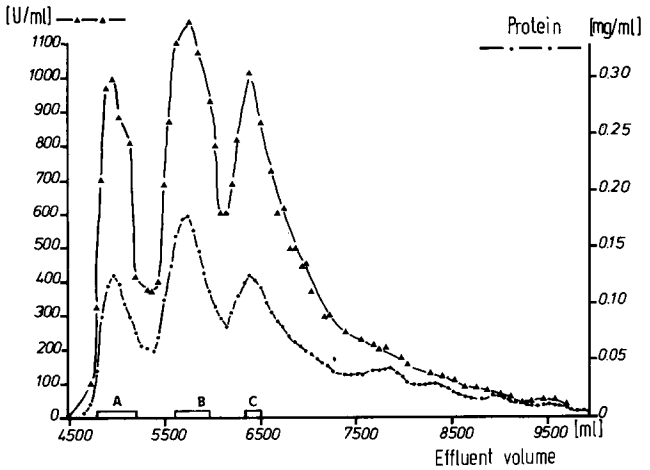

Fig. 1. DEAE-cellulose chromatography from human skeletal muscle triosephosphate isomerase. Activity and protein were measured and the peaks pooled as shown.

Results

Isolation of the multiple forms

Employing a shallow NaCl gradient, three protein and corresponding enzyme activity peaks were eluted. Peak fractions were pooled as indicated by the letters A, B, and C (Fig. 1). In cellogel electrophoresis with Coomassie, as well as with activity staining, the B and C forms contain very small amounts of the other forms. The A form shows no contamination (Fig. 2). From the elution profile of the cellulose column, an activity ratio of 25 : 46 : 29 (% of total activity) for A : B : C was estimated. The molecular weight of the triosephosphate isomerase subunits was determined in polyacrylamide electrophoresis in the presence of 0.1% sodium dodecyl sulfate. All three forms showed the same subunit M_r 26 500—27 300 which was identical with the subunit of rabbit muscle triosephosphate isomerase [23,5]. Using the amino acid sequence of the rabbit enzyme, Corran and Waley [24] computed a molecular weight of 26 600.

Amino acid analysis

Based on 496 known amino acids from the sequence of rabbit triosephosphate isomerase [24], the composition of amino acid residues per mol human triosephosphate isomerase was calculated (Table II). The amino acid composition of the three forms revealed small, insignificant differences. The spectrophotometric determination of tryptophan and thiol groups yielded identical numbers for the three forms (Table II).

Reversible dissociation

While the A and C forms remained unchanged in their electrophoretic

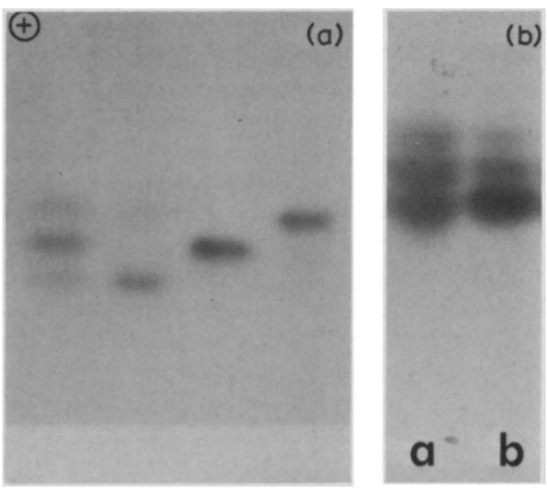

Fig. 2. a. Cellogel-electrophoresis of the isolated subforms of human skeletal muscle triosephosphate isomerase. The gel was stained with Coomassie brilliant blue. The isolated triosephosphate isomerase before separation on DEAE-cellulose and the subforms, A, B, and C are shown. For each subform about 2.4 to 4.8 μg were applied to the gel. b) Triosephosphate isomerase forms in (a) normal blood, and in (b) enriched reticulocytes (22.5%). From each blood sample, 60 mU triosephosphate isomerase were applied and the gel was stained for activity [18].

TABLE II

AMINO ACID COMPOSITION OF THE THREE MAIN FORMS OF TRIOSEPHOSPHATE ISOMERASE

The data for the A, B and C subforms represent 4, 9 and 9 analyses, respectively, with the standard deviation. The amino acid residues were calculated for a total of 496 amino acids.

Amino acids	Triosephosphate-isomerase subforms		
	A	B	C
Aspartic acid	38.9 ± 0.21	40.0 ± 0.95	40.8 ± 0.71
Threonine	27.8 ± 0.17	28.0 ± 0.30	27.6 ± 0.30
Serine	24.4 ± 0.23	24.5 ± 0.27	24.2 ± 0.28
Glutamic acid	51.4 ± 1.59	49.7 ± 0.88	51.9 ± 0.78
Proline	23.0 ± 0.45	21.6 ± 0.64	21.5 ± 0.58
Glycine	48.8 ± 0.25	47.7 ± 0.89	47.9 ± 0.36
Alanine	54.0 ± 0.61	52.7 ± 0.64	52.1 ± 0.50
Valine	55.0 ± 0.42	55.6 ± 0.24	54.8 ± 0.34
Methionine	3.9 ± 0.42	4.4 ± 0.26	4.3 ± 0.17
Isoleucine	29.5 ± 0.26	29.6 ± 0.33	29.4 ± 0.46
Leucine	31.8 ± 0.46	34.4 ± 0.57	32.6 ± 0.71
Tyrosine	8.3 ± 0.36	8.6 ± 0.17	8.3 ± 0.23
Phenylalanine	16.2 ± 0.40	16.6 ± 0.18	16.0 ± 0.35
Histidine	7.6 ± 0.21	7.8 ± 0.69	7.2 ± 0.45
Lysine	39.4 ± 1.05	39.5 ± 0.43	40.8 ± 0.69
Arginine	15.9 ± 0.31	16.5 ± 1.07	15.9 ± 0.54
Tryptophan [a]	9.82	9.94	10.0
Cysteine [b]	9.96	10.2	9.32

[a] Determined spectrophotometrically according to Edelhoch [21], mean of two determinations.
[b] Determined spectrophotometrically with DTNB [20], mean of two determinations.

behavior after reversible dissociation, the B form dissociated and re-associated into the original A, B and C forms. These hybridization experiments suggest that the triosephosphate isomerase has two different subunits, coded as α and β. The subunit composition of the three forms A, B and C is $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ respectively.

Antibody titration

Antibodies (antiserum I) prepared against unresolved human triosephosphate isomerase formed a single line of precipitation with the unresolved triosephosphate isomerase and the three separate forms in Ouchterlony immunodiffusion. The precipitation lines fused completely, showing the presence of identical determinants in the three forms. The same result was obtained with antiserum II against the pure A form. In the Mancini test, only one ring of precipitation was visible when the three forms were mixed, indicating antigenic identity.

Triosephosphate isomerase was inactivated by association with antibody. If the determinants were the same for all forms, they should be inactivated to the same degree by increasing amounts of antibody. For all three forms, the ratio of the antisera I and II required to inhibit a given amount of units was the same (Table III).

Kinetic properties

The specific activities (U/mg protein) of the A and B forms were identical.

TABLE III

KINETIC PROPERTIES

Property	n	Unresolved enzyme	Triosephosphate isomerase forms					
			A		B		C	
Distribution in %	2	100 (100)	24—26	(5—10)	45—47	(70—75)	28—30	(20—25)
Specific activity; U/mg	3	9200	8400		8300		7700	
U/ml Antiserum I	1	500	550		540		540	
U/ml Antiserum II	3	510	510		510		470	
K_m Glyceraldehyde phosphate (μM)	5	13.6	13.1	(11.2)	13.3	(14.5)	13.0	(18.4)
K_m Dihydroxyacetone phosphate (μM)	2	185	180	(82.5)	170	(42.5)	185	(38.2)
pH Optimum (100% activity)	1		5.6—8	(7.8)		(7.8)	5.6—8	(7.8)
Heat stability at 60°C $T_{1/2}$ (min)	3	37	34		37		37	
Activation energy (KJ/mol)	1		41.9				42.3	

The values given in parentheses are taken from Gracy [27].

The C form had a somewhat lower specific activity, which correlates with the lower content of free sulfhydryl groups in this form. The original purified enzyme had a higher specific activity than the individual subforms (Table III). At this time, we assume that the differences among the subforms and the unresolved mixture are the result of partial inactivation due to the additional steps employed in their isolation and not due to a specific catalytic property. The same phenomenon was observed in rabbit muscle triosephosphate isomerase [5].

No essential differences in the heat stability at 60 °C of the three forms were found (Table III). The Michaelis-Menten constants for D-glyceraldehyde 3-phosphate and dihydroxyacetonephosphate were identical for all three forms. Hartman et al. [28] observed that the K_m value varies markedly with ionic strength. A possible explanation for this phenomenon has been offered by Campbell et al. [29]. In our studies, differing dependence of the K_m apparent upon the ionic strength of the buffer was observed for the three forms. As Fig. 3 displays, C showed the least dependence, A the greatest, and B was intermediate (not shown).

The inhibition by phospho*enol*pyruvate, α-glycerolphosphate, 2-phosphoglycollate and phosphate was tested with an inhibitor concentration of 1 and 2.5 mM. In addition, the inhibition by folic acid at concentrations of 0.26 and 0.52 mM was determined. None of the inhibitors, however, exhibited a differing effect on the three forms.

Electrophoretic studies

After lengthy storage of nearly a year in 3.5 M $(NH_4)_2SO_4$ (pH 8.0), the purified forms showed additional bands in the electrophoresis, which had not been observed shortly after isolation. Form A showed a major band, C′, plus a faint intermediate band, B′, and form B showed an additional band, D′. Form C exhibited two further scarcely visible bands, D′ and E′. After incubation of form A in 0.1 M potassium phosphate buffer (pH 6.0), the additional bands B′ and C′ disappeared.

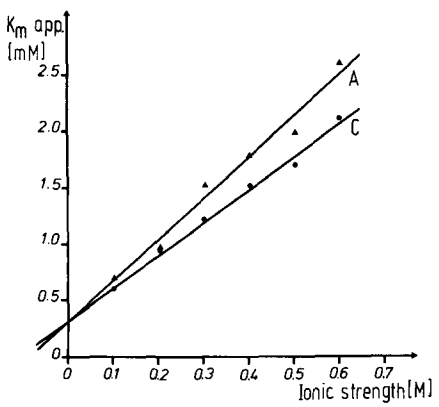

Fig. 3. The dependence of the apparent K_m on the ionic strength. In 0.1 M triethanolamine-HCl buffer (pH 7.8), the ionic strength was increased by NaCl and the corresponding K_m values were measured. The intermediate B form was deleted to not confuse the diagram.

Granulocytes were isolated as previously described [18]. The electrophoresis showed a predominant A band and a weak B band with approx. 10% of the total activity. T-lymphoblasts, B-lymphoblasts, Burkitt lymphoblasts, as well as freshly isolated lymphocytes, all showed a dominant A form. The B form was stronger than in the electrophoresis of the granulocytes and a weak C band was also visible. If greater amounts of extract were applied on the gel, a very weak D band was observed, as in skeletal muscle. Peters et al. [11] demonstrated two bands, A and B, in white blood cells, again with the appearance of an E band when greater quantities of triosephosphate isomerase were applied, and/or when long staining periods were allowed. In contrast, Kester et al. [14] found only one band in fresh lymphocyte extract applied on polyacrylamide gel, and an additional band in cultured lymphoblasts.

Fibroblasts showed only two bands, A and B, independent of the amount of extract applied. Peters et al. [11] observed two equally strong bands, A and E, and a weak B band. At this time, we cannot explain why we did not observe an E band in cultured fibroblasts.

A reticulocyte-rich fraction with 22.5% reticulocytes was gained by the elutritration method [30]. Compared with normal blood (Fig. 2b), this fraction shows a noticably greater amount of A and a lower B and C content.

Discussion

Triosephosphate isomerase was isolated in several batches from skeletal muscle. In SDS-polyacrylamide gel electrophoresis, the purified enzyme is homogeneous and exhibits only one band which has the same electrophoretic mobility as the rabbit, chicken and yeast enzymes. In cellogel electrophoresis, the native dimeric enzyme showed three main forms (which share about 97% of the total activity), and two minor forms. This was the same pattern as has already been observed for the purified rabbit muscle enzyme [5].

The purified human triosephosphate isomerase was resolved in three principal forms (A, B and C) by chromatography on DEAE-cellulose (Fig. 1). The tail of the elution profile contains two minor forms (D and E), which comprise approx. 3% of the total activity, and which have, at this time, not been separated. The additional F, G and H forms could not be detected. We assume that due to the long staining time (11), the minor forms, D to H, appear with such strong intensity in the starch gel electrophoresis. Therefore, the staining intensity of the main bands was not proportional to the triosephosphate isomerase activity and the minor forms were overestimated. From our cellogel electrophoresis with different amounts of enzyme, we conclude that the F, G, and H forms together represent distinctly less than 1% of the total triosephosphate isomerase activity. However, we cannot exclude the possibility that these minor forms were selectively lost in the isolation procedure.

The amino acid analysis of the three forms showed no significant differences (Table II). This is in accordance with the analysis of the multiple forms of the rabbit muscle triosephosphate isomerase [5], but is contrary to Sawyer et al. [10], who found considerable differences between the three main forms isolated from human erythrocytes by isoelectrofocusing. According to their analysis, the differences in the amino acid composition between the

homodimeric A and C forms are greater than the differences between the coelacanth and rabbit triosephosphate isomerase (Table IV). This information would allow one to extrapolate that the gene should have been duplicated already $3.5 \cdot 10^8$ years ago. However, the coelacanth and chicken have only one electrophoretic form and one sequence. For the rabbit muscle enzyme, Corran and Waley [24] likewise obtained only one sequence and assumed that the multiple forms were most probably the consequence of secondary modifications, e.g. deamination of sensitive amides. Therefore, it is most likely that also for the human triosephosphate isomerase only one polypeptide chain is primarily synthesized. This is supported by our amino acid analysis. The normal banding pattern in 50% triosephosphate isomerase deficiencies could also be best explained by this assumption [18].

In tissues with a short lifetime, such as leucocytes, lymphoblasts and fibroblasts and fibroblasts, a strong A band, a weak B band and a hardly visible C band could be detected. We assume, therefore, that the A form is primarily synthesized and is then transformed into the C form, a process which occurs with cell aging and might depend on the hydrogen ion concentration and ionic strength. This assumption is supported by the spontaneous transformation of form A to C′ when stored in basic solution with a high ionic strength over a long period of time. Similar observations were made by Peters et al. [11]. When fresh extracts of cultured fibroblasts which had shown only the A and E forms were stored for several days at 4°C, the 'isozymes' in positions B, C and D frequently appeared, suggesting that these 'isozymes' may be derived from 'iso-

TABLE IV

COMPARISON OF THE AMINO ACID COMPOSITION OF TRIOSEPHOSPHATE ISOMERASE FROM DIFFERENT SOURCES

Amino acid	Coela- * canth [26]	Chicken * [25]	Rabbit * [24]	Human ** A & C present study	Human A ** [10]	Human C ** [10]
Asp	18	19	21	20	30	30
Thr	11	10	15	14	10	10
Ser	13	12	12	12	23	16
Glu	29	26	27	26	25	26
Pro	10	7	10	11	16	16
Gly	26	27	24	24	21	27
Ala	23	28	27	26	20	21
Val	28	23	25	28	18	15
Met	2	2	2	2	2	2
Ile	12	17	15	15	8	7
Leu	17	17	15	16	20	24
Tyr	5	4	4	4	6	7
Phe	10	8	8	8	9	10
Lys	21	23	21	20	15	15
His	4	8	4	4	7	4
Arg	8	8	8	8	8	9
Trp	5	5	5	5	5	4
Cys	5	4	5	5	5	5

* From the sequence

** From the amino acid analysis, the nearest integer is given.

zyme' A. It was also noted that after storage, the activity of 'isozyme' E diminished progressively [11]. The D and E forms of the rabbit muscle enzyme were converted by a freezing-thawing procedure into the three main forms and into additional forms up to J [5]. A further support for posttranslational alterations of human triosephosphate isomerase was offered by Turner et al. [15], who found that the electrophoretic banding pattern of triosephosphate isomerase from erythrocytes was changed with age, something we too were able to confirm.

The kinetic properties of the erythrocyte enzyme were determined by Gracy [27], who found that human A form had a higher affinity for D-glyceraldehyde 3-phosphate and a lower affinity for dihydroxyacetone phosphate than the C form. The B form was intermediate. We could not confirm these findings. From a comparative characterization of various parameters affecting the activity of the enzyme (Table III), the three forms A, B, and C can be classified as 'isokinetic', as has already been established for the rabbit muscle triosephosphate isomerase forms [5]. Therefore, it remains obscure whether the multiple forms have any physiological significance or are just a normal product of the aging of this protein.

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