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ISOLATION OF BUFFALO MUSCLE ALDOLASE AND COMPARISON OF ITS PROPERTIES WITH THOSE OF RABBIT MUSCLE ALDOLASE

S. TAZEEN PASHA * and A. SALAHUDDIN **

Protein Research Laboratory, Department of Biochemistry, J.N. Medical College, A.M.U., Aligarh 202001 (India)

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Summary

Fructose-1,6-bisphosphate aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) was isolated from buffalo muscle by fractionation with ammonium sulphate and subsequent purification by phosphocellulose column chromatography using a linear salt gradient. As judged by gel filtration and electrophoresis in polyacrylamide gel, the enzyme was homogeneous with respect to size and charge. The molecular weight and Stokes radius of the enzyme were determined from its elution profile on a calibrated Sephadex column and the respective values were 162000 and 4.55 nm. The diffusion coefficient and frictional ratio were computed to be $4.8 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1} and 1.27, respectively. The molecular weight of the polypeptide chain as measured by sodium dodecyl sulphate polyacrylamide gel electrophoresis was 40750. This taken together with the native molecular weight suggested a four-subunit model for the protein. The N- and C-terminal residues of polypeptide chains were identified to be proline and tyrosine, respectively. At pH 8.0 the Michaelis-Menten constant and maximum attainable velocity were found to be 8.1 μM and 27 μM Fru-1,6-P_2 split/min per mg, respectively. The buffalo muscle aldolase was found to be similar to rabbit muscle aldolase in physicochemical properties. However, the two enzymes differ significantly in pH optimum; the pH optima of the buffalo and rabbit enzymes were determined under identical conditions to be 8.0 and 8.6, respectively.

Introduction

Fru-1,6-P_2 aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) is distributed in micro-organisms [1—3], plants [4]

* Present address: National Institute of Communicable Diseases, Delhi.
** To whom to address correspondence.

and mammalian tissues [5]. The enzyme exhibits interspecies differences in molecular and biological properties [6—8]. Within the same animal species aldolase of one tissue may be different from that of another. Thus, three forms of aldolase, namely, A, B and C have been detected, respectively, in muscle, liver and brain tissues. They are distinguishable in catalytic properties, electrophoretic mobility, chromatographic profile, amino acid composition and peptide mapping [9,10]. From kinetic parameters [11] it appears that aldolase A is primarily involved in the cleavage of Fru-1,6-P_2 in glycolysis, whereas the B form is active in gluconeogenic condensation of triose phosphates and in the cleavage of fructose 1-phosphate; no specific biological function of aldolase C has yet been apparent. In view of these inter- and/or intraspecies variations in physical as well as enzymatic properties of Fru-1,6-P_2 aldolase, it would be interesting to study enzymes from different sources. In this paper we report the isolation, purification and characterization of Fru-1,6-P_2 aldolase from a hitherto uninvestigated source, i.e., buffalo muscle.

Materials and Methods

Cytochrome *c*, α-chymotrypsinogen, ovalbumin (grade V), bovine serum albumin, pepsinogen, alkaline phosphatase, carboxypeptidase-A, catalase, rabbit muscle aldolase, aldolase calibration solution, pyridoxal-5′-phosphate, Coomassie Blue and 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) were purchased from Sigma Chemical Co., U.S.A. Papain, phosphocellulose and barium salt of Fru-1,6-P_2 were obtained from V.P. Chest Institute, Delhi. The latter was converted to the sodium form before use. Reagents used in Sephadex gel filtration and in polyacrylamide gel electrophoresis were the same as previously described [12]. Other chemicals were of reagent grade.

Isolation of buffalo muscle aldolase

Following Taylor [13] 250 g minced buffalo muscle was extracted with a solution containing 1 mM EDTA, 0.01 M Tris · HCl, pH 7.4. The extract was brought to 40% ammonium sulphate saturation and allowed to stand for 6 h. The supernatant was then brought to 60% saturation and the precipitated aldolase was collected. The precipitate was redissolved in 0.01 M Tris · HCl, pH 7.4, containing 1 mM EDTA, and dialysed extensively against the same buffer to remove ammonium sulphate. Some insoluble protein material which settled down in the dialysis bag was removed by centrifugation. Aldolase was further fractionated by chromatography on phosphocellulose column using a linear NaCl gradient (0.01—0.5 M).

Measurement of enzyme activity

The activity of buffalo muscle aldolase was measured in 0.01 M Tris · HCl buffer, pH 8.0 by the method of Sibley and Lehninger [14]; the pH optimum for the buffalo enzyme was found at pH 8.0. In 1.9 ml, 120 μg of the enzyme was first taken with 56 mM hydrazine sulphate. To this was added 0.1 ml of 0.08 mM Fru-1,6-P_2 (final concentration 20 μM) and the solution was incubated for 30 min at 37°C; the reaction was stopped by adding 2 ml of 20% trichloroacetic acid. To 1 ml of this solution was added 1 ml of 0.75 M NaOH

and after 15 min 1 ml of 0.1% 2,4-dinitrophenyl hydrazine in 2 M HCl was added and the solution incubated at 37°C for 20 min. Then 7 ml of 0.75 M NaOH was added and the colour intensity measured at 540 nm against the control. The latter was prepared in an identical manner except that Fru-1,6-P_2 was added after the addition of trichloroacetic acid. Corresponding to the measured absorbance, activity was read from aldolase calibration curve prepared with aldolase calibration solution. The specific activity was expressed as μM of Fru-1,6-P_2 cleaved/min per mg of the enzyme. It should be pointed out that the enzyme followed Michaelis-Menten kinetics under the conditions of our kinetics experiments, and that except in the determination of Michaelis-Menten constant, the concentration of the substrate relative to that of the enzyme was sufficient to saturate the enzyme.

Gel filtration

Molecular weight and hydrodynamic properties of buffalo muscle aldolase were determined in 0.02 M sodium phosphate buffer, pH 7.0 by Sephadex G-100 column chromatography as has already been reported [15]. The marker proteins with their molecular weights [16] and Stokes radii [17] in parentheses were ovalbumin monomer (43 000, 2.73 nm); bovine serum albumin monomer (69000, 3.55 nm); alkaline phosphatase (80000, 3.9 nm); ovalbumin dimer (86000) and catalase (240000, 5.2 nm). The void volume, V_o, the inner volume, V_i, and the total volume, V_t, of the column as well as elution volume for a given protein were determined by standard procedures [17]. The distribution coefficient, K_d, the available distribution coefficient, K_{av}, were then calculated [17] for each marker protein as well as for the aldolase.

Electrophoresis

Polyacrylamide gel electrophoresis of rabbit and buffalo muscle aldolase was carried out at pH 8.6 in 0.02 M Tris/glycine buffer by the method essentially due to Davis [18]. The subunit molecular weight of aldolase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis performed by the method of Weber and Osborn [19] as described earlier [15]. The marker proteins with their molecular weights in parentheses were cytochrome *c* (11700), papain (23400), α-chymotrypsinogen (25700), pepsinogen (40000), ovalbumin (43000) and bovine serum albumin (69000). The relative mobility of sodium dodecyl sulphate · protein complex in the polyacrylamide gel was determined both for marker proteins as well as for the aldolase by dividing the mobility of the protein by that of cytochrome *c*. The measured values of R_m for marker proteins were: cytochrome *c*, 1.0; papain, 0.65; α-chymotrypsinogen, 0.62; pepsinogen, 0.38; ovalbumin, 0.35; and bovine serum albumin, 0.13.

Ultraviolet light absorption and fluorescence measurements were made on Carl Zeiss spectrophotometer and Aminco-Bowman spectrophotofluorometer, respectively. Protein concentration was determined by the method of Lowry et al. [20] or by dry weight method [21]. The N-terminal residue of buffalo muscle aldolase was determined by the method of Gray [22] using dansyl chloride. The C-terminal residue was identified by the standard procedure [23] using carboxypeptidase-A. The amino acids were identified as before [24] by thin layer chromatography.

TABLE I

ISOLATION AND PURIFICATION OF BUFFALO MUSCLE ALDOLASE

Extract	Protein (g)	Specific activity (μM of Fru-1.6-P_2 split/min per mg)	Purification (-fold)
Extract at 0% $(NH_4)_2SO_4$ saturation	5.800	0.2	0
60% $(NH_4)_2SO_4$ saturation precipitate	0.815	8.0	40
Phosphocellulose fraction F_3	0.320	25.5	128

Results

The amount of protein extracted from 250 g of buffalo muscle aldolase was 5.8 g. The stages of purification are summarized in Table I. The enzyme obtained at 60% ammonium sulphate saturation contained at least four protein.

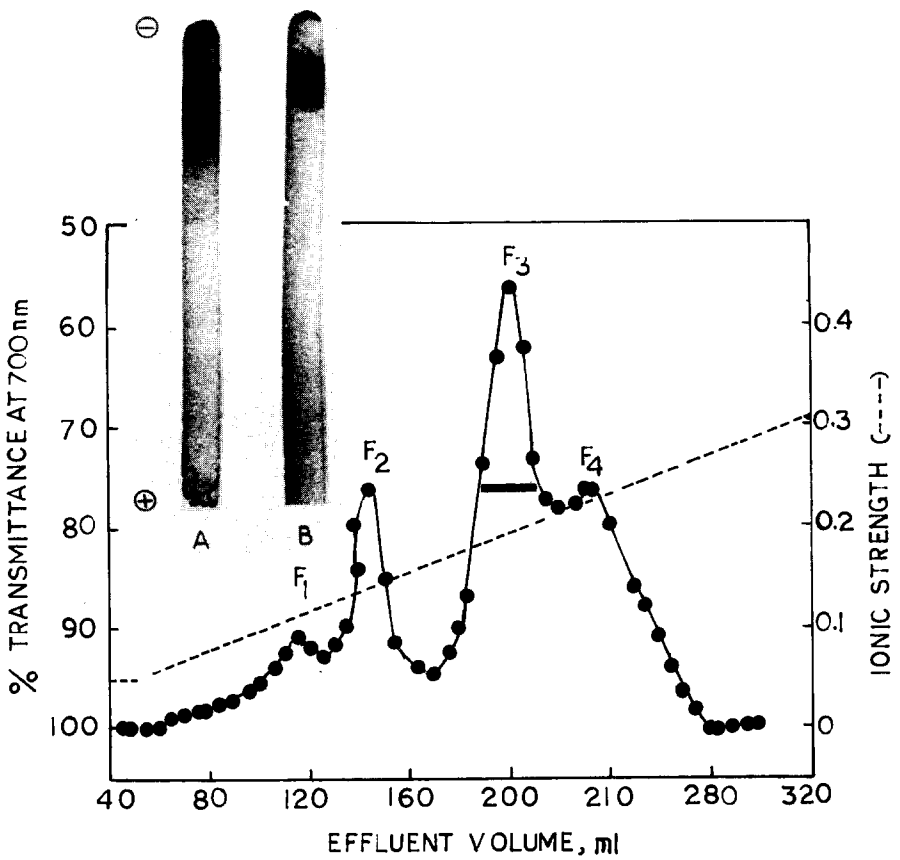

Fig. 1. Chromatography of buffalo muscle aldolase on phosphocellulose column. The column (1.6 cm × 23 cm) was equilibrated with Tris · HCl buffer; pH 7.4, ionic strength 0.01. About 30 mg protein in 2.0 ml Tris · HCl buffer, pH 7.4 was applied on the column. The elution was performed in 5.0-ml fractions at a flow rate of 20 ml/h using a linear sodium chloride gradient from 0.01 to 0.5 M. The broken line shows the gradient. The protein fractions under the thick line were pooled and used throughout. The inset shows polyacrylamide gel (7.5%) electrophoresis of buffalo muscle aldolase (A) and rabbit muscle aldolase (B) at pH 8.6 in 0.02 M Tris/glycine buffer.

fractions which were obtained by phosphocellulose column chromatography using a linear sodium chloride gradient from 0.01—0.5 M (see Fig. 1). The protein fractions under peak F_3 were pooled as indicated in Fig. 1. The protein had Fru-1,6-P_2 cleavage activity and was found to be homogeneous with respect to size (single symmetrical peak in Sephadex gel chromatography) and charge (single band in polyacrylamide gel electrophoresis; see Fig. 1). When stored at 4°C in water, the enzyme retained its full activity for three weeks, after which some loss of activity was noticed; the inactivation was more significant when the solution of the enzyme was dilute. When stored at 4°C in presence of ammonium sulphate, the activity of the enzyme remained unaltered up to about two months. The effect of the substrate concentration in the range 0.02—5 μM on enzyme activity was investigated at pH 8.0. Michaelis-Menten constant, K_m, as determined from linear least squares fit of the data to double-reciprocal plot [25] was 8.1 μM; the value of the maximum attainable velocity, V, was 27 μM of Fru-1,6-P_2 split/min per mg. The pH activity profile of the enzyme obtained in different buffers was bell-shaped with a maximum occurring at pH 8.0. For a given pH the dependence of enzyme activity on the nature of buffer was found to be insignificant under the conditions of our experiments. Proline and tyrosine were qualitatively identified to be the N- and C-terminal residues of each of the polypeptide chains of buffalo muscle aldolase.

In 0.01 M Tris · HCl buffer, pH 7.4 the enzyme absorbed maximally at 278 nm in the ultraviolet region. The specific extinction coefficient, $E^{1\%}_{1cm}$, was determined by measuring absorbance of a series of aldolase solutions of known concentration and its values at 255, 278 and 280 nm were 8.5, 9.7 and 9.3 respectively. The difference spectrum of aldolase (1 mg/ml) plus 50 μM Fru-1,6-P_2 measured against the enzyme alone (1 mg/ml) showed, among others, one trough at 285 nm and two shoulders near 283 and 290 nm. These features in the difference spectrum strongly suggest that the environment around aromatic chromophores (particularly tyrosine and tryptophan residues) of the enzyme was significantly altered due to the conformational change induced by Fru-1,6-P_2 and/or its cleaved products. This is further supported by our fluorescence results. With 278 nm as the excitation wavelength, the emission maximum of the enzyme in 0.01 M Tris · HCl buffer, pH 7.4 was found to be 346 nm. The latter is the characteristic of the tryptophan containing protein. Interestingly, the fluorescence intensity of the enzyme (0.4 mg/ml) was markedly (46%) reduced by 50 μM Fru-1,6-P_2. These findings are similar to those previously reported for rabbit muscle aldolase. The quenching of the tryptophan fluorescence by substrate [26] and alteration in the native enzyme conformation due to its interaction with the substrate [27—32] are known.

Following well known empirical and theoretical relations [16,33,34], the gel filtration data obtained on the marker proteins, described in the Materials and Methods section, were analysed by the method of least squares, yielding the following straight-line equations:

$$V_e/V_o = 5.735 - 0.844 \log M_r \quad (1)$$

$$M_r^{1/3} = 101.7 - 85.98\ K_d^{1/3} \text{ (Porath [33]} \quad (2)$$

$$(-\log K_{av})^{1/2} = 0.189\ a - 0.028 \text{ (Laurent and Killander [34]} \quad (3)$$

$$\text{erfc}^{-1} K_d = 0.264\, a - 0.243 \text{ (Ackers [16]} \quad (4)$$

where a is the Stokes radius of a given protein.

The values of V_e, V_e/V_o, K_{av} and K_d for buffalo muscle aldolase were measured to be 129 ml, 1.317, 0.19 and 0.17, respectively. The molecular weight of buffalo muscle aldolase comes out to be 162180 from Eqn. 1 and 161320 from Eqn. 2; the average value is 162000. The Stokes radius is 4.6 nm from Eqn. 3, and 4.5 nm from Eqn. 4; the average Stokes radius is 4.55 nm. The diffusion coefficient and frictional ratio, f/f_o, of the enzyme were calculated from gel filtration data corresponding to the Stokes radius of 4.6 nm by the help of the following equations:

$$D = kT/6\pi\eta a \quad (5)$$

$$f/f_o = a/(3\bar{v}_2 M_r/4\pi N)^{1/3} \quad (6)$$

where k is Boltzmann constant, η, is the viscosity in poise (0.01 p), T is the absolute temperature (303° K), $\bar{v}_2$ is the partial specific volume and was assumed to be the same (0.747 ml/g) as for rabbit muscle aldolase [35], M_r is the molecular weight (162000). The values of D and f/f_o for buffalo muscle aldolase were calculated from Eqns. 5 and 6 to be $4.8 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1} and 1.265, respectively. The curve between relative mobility, R_m, of a given protein in sodium dodecyl sulphate polyacrylamide gel electrophoresis and logarithm of its molecular weight was found to be linear. A least squares analysis of the results on log M_r and R_m yielded the straight line which fits the equation,

TABLE II

COMPARISON OF THE PROPERTIES OF BUFFALO AND RABBIT MUSCLE ALDOLASES

Property	Rabbit enzyme	Buffalo enzyme
Maximum attainable velocity in 0.01 M Tris · HCl, pH 8.0 (μM Fru-1,6-P_2 split/min per mg)	25.0	27.0
K_m for Fru-1,6-P_2 (μM) in 0.01 M Tris · HCl buffer, pH 8.0	9.2	8.1
Optimum pII for enzyme activity	8.6	8.0
Percent inhibition by 0.4 mM pyridoxal 5'-phosphate, pH 8.0	76.0	70.0
N-terminal residue	Proline [b]	Proline
C-terminal residue	Tyrosine [b]	Tyrosine
λ_{max} in 0.01 M Tris · HCl, pH 7.4 (nm)	278	278
$E^{1\%}_{1cm}$ in 0.01 M Tris · HCl, pH 7.4 (cm$^2 \cdot$ g^{-1})	9.1	9.3
λ_{max} for excitation, pH 7.4 (nm)	278	278
λ_{max} for emission fluorescence, pH 7.4 (nm)	346	346
Molecular weight under native conditions	158 000 [a]	162 000
Molecular weight under denaturing conditions	40 000 [a]	41 000
Stokes radius of the native enzyme (nm)	4.5 [b]	4.55
$10^7 \times$ diffusion coefficient under native condition (cm$^2 \cdot$ s^{-1})	4.63 [b]	4.8
f/f_0 of native enzyme	1.26 [b] 1.31 [b]	1.27

[a] Ref. 35.
[b] Ref. 5.

$$\log M_r = 4.95 - 0.883\, R_m \quad (7)$$

For buffalo muscle aldolase, R_m was measured to be 0.38 which according to Eqn. 7 would correspond to a molecular weight of 40750.

The comparison of molecular and enzymatic properties of buffalo muscle aldolase and rabbit muscle aldolase was made under identical experimental conditions. The results are summarized in Table II which also includes some published data obtained earlier on rabbit muscle aldolase [5]. From Table II it can be seen that small but significant differences in enzymatic properties were noted under identical experimental conditions. It is to be noted that the experimental uncertainty involved in the determination of V, optimum pH and percent inhibition by pyridoxal 5′-phosphate were found to be generally below 5%; the error in the determination of K_m was near 15%. Thus, the observed differences between the two enzymes in V, optimum pH, and percent inhibition by pyridoxal 5′-phosphate seem to be experimentally significant, whereas the observed difference in K_m values is more apparent than real. The significant qualitative conclusion which can be deduced from a close examination of the pH-activity profile is that two ionizable groups with pK values near pH 7.2 and 8.5 may be involved in the enzymatic cleavage of Fru-1,6-P_2 by buffalo muscle aldolase.

Discussion

The procedure used in the isolation of Fru-1,6-P_2 aldolase from buffalo muscle yielded a pure and homogeneous preparation of the enzyme. The hydrodynamic paramters, i.e., diffusion coefficient ($4.8 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1}) and frictional ratio (1.27) calculated from gel filtration data suggest that the overall native conformation of buffalo muscle aldolase is compact and globular. The enzyme seems to possess similar physicochemical properties as found for rabbit muscle aldolase. Thus, both enzymes possess identical N- and C-terminal residues; λ_{max} for the ultraviolet absorption and the emission fluorescence; and within experimental error, $E^{1\%}_{1cm}$ at 278 nm. Likewise, the molecular weight, Stokes radius and other related hydrodynamic parameters, e.g., diffusion coefficient and frictional ratio, were found to be identical for the two enzymes. In addition, preliminary results on immunodiffusion using the two enzymes and the rabbit antiserum raised against buffalo enzyme also showed that the buffalo and the rabbit enzymes probably possess similar overall native conformation. The fact that the molecular weight of buffalo muscle aldolase as measured by gel filtration under native conditions was 3.95 times the molecular weight of the individual polypeptide chain of the enzyme determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis, strongly suggests that buffalo enzyme consists of four subunits. Earlier studies [11,35] have unequivocably demonstrated the four subunit model of rabbit muscle aldolase. In addition, the data indicated that the native conformation of buffalo muscle aldolase was significantly altered by the substrate Fru-1,6-P_2 and/or its cleaved products. This has also been noticed for rabbit muscle aldolase on several occassions [26—32]. Strikingly, the two enzymes with similar Michaelis-Menten constant for the Fru-1,6-P_2 cleavage activity, differ in other enzymatic properties, more

importantly in pH optimum. The pH optimum for rabbit muscle aldolase noted here as well as elsewhere [14] is near 8.6, whereas that of the buffalo enzyme was found to be 8.0.

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