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ANALYSIS OF TRANSKETOLASE AND IDENTIFICATION OF AN ENZYME VARIANT IN HUMAN LEUKOCYTES

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SUMMARY: Human leukocyte transketolase of fresh cell extracts has been analyzed by isoelectrofocusing on agarose gels (pH 3-10). The enzyme was then transblotted on nitrocellulose and detected with specific anti-transketolase IgG coupled to an avidin/biotin-immunoperoxidase system. Each sample yielded multiple enzyme forms, within a pI range of about 7.4-8.4. Transketolase profile, however, was not identical in all extracts. There are two mainly distinct patterns, showing qualitative and quantitative differences: a standard profile, which is predominant, and a variant, found in three unrelated subjects out of the two hundred and twenty. Standard and variant enzyme have similar Km values for ribose 5-P and xylulose 5-P and the same mobility on SDS-PAGE.

Transketolase (TK) (EC 2.2.1.1.) is an enzyme of the non-oxidative pentose cycle sequence and it catalyzes the transfer of a two-carbon atom moiety (1) from suitable ketol-donor and aldo-acceptor sugars. The enzyme requires thiamine pyrophosphate as essential cofactor for activity (1-3), therefore it has been studied as a marker of several pathological conditions linked to thiamine deficiency (4). Alcoholism (5), Wernicke-Korsakoff syndrome (6,7), Alzheimer's disease (8) and other neuropathies have been reported to induce transketolase abnormalities of either kinetic properties or isoenzyme pattern.

The presence of multiple forms of TK as well as the occurrence of enzyme variants are still controversial. So far human erythrocyte TK has alternatively been described either as an

Abbreviations used: Transketolase (TK); isoelectric focusing (IEF); D-ribose 5-phosphate (R5P); D-xylulose 5-phosphate (Xu5P); nitrocellulose (NTC); phosphate buffered saline (PBS); Mono-Poly Resolving Medium (M-PRM).

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heterogeneous (9,10) or homogeneous protein family (11). Moreover, the identification of several enzyme variants by Kaczmarek and Nixon (9) has not been confirmed by Kaufmann et al. (10). These discrepancies are probably due to the low reliability of the procedure employed which consists of partial purification and isoelectrophoretic (IEF) separation of erythrocyte TK, eventually detected with a fairly specific stain for activity (12).

In this study we also used IEF for TK separation but all other conditions and the starting material were changed. First of all human leukocytes, which are a much richer source of TK than red cells, have been employed. Secondly, the enzyme was analyzed directly in fresh cytosolic extracts without further manipulation. Thirdly, transketolase detection was accomplished by specific anti-TK antibodies to the pure human leukocyte enzyme (13). Results obtained with peripheral blood leukocytes confirm TK heterogeneity and demonstrate with an immunological method the occurrence of at least two enzyme variants in humans.

MATERIALS AND METHODS

Cell harvesting and extracts. Heparinized whole blood samples (5 ml) from healthy donors were centrifuged at 800 g for 30 min on Mono-Poly Resolving Medium (Flow Laboratories Ltd.). Mononuclear and polymorphonuclear leukocyte fractions were recovered and washed with PBS. Occasionally, 0.87 % NH_4Cl was used to eliminate contaminating red cells. Packed cells were then suspended with 0.5 ml of 10 mM Tris-HCl buffer pH 7.4, containing 0.5 mM phenylmethylsulphonyl fluoride, and disrupted by sonication for 2 min in ice at 100 W (Labsonic sonicator, Melsungen, West Germany). Lysates were then centrifuged at 100,000 g for 20 min at 4° C (Beckman TL-100, Ultracentrifuge) to collect cytosolic extracts.

Reagents. Acrylamide, bisacrylamide, Isogel agarose-EF, nitrocellulose membrane (0.45 µm) and TEMED were purchased from LKB (Bromma, Sweden). Thiamine pyrophosphate, D-xylulose 5-phosphate, D-ribose 5-phosphate, bovine serum albumin, phenylmethylsulphonyl fluoride, N,N-dimethyl formamide and 3-amino-9-ethylcarbazole were from Sigma Chemical Company (St. Louis, Missouri). Auxiliary enzymes of TK assay mixture and NADH were from Boehringer Mannheim (West Germany). Specific anti-TK IgG were obtained by affinity chromatography of rabbit immune sera (13) on Protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden).

Assays. TK activity was assayed spectrophotometrically at 37° C, as previously reported (14). One unit corresponded to the amount of enzyme that catalyzes the oxidation of 1 µmol of NADH, at 37°C in a cuvette (1 cm of light path) in the presence of 2 mM R5P and 1 mM Xu5P. Proteins were determined according to Bradford (15).

IEF and SDS-PAGE analyses. Isoelectric focusing (IEF) was carried out in a Multiphor LKB 2217 apparatus connected with a LKB 2297 constant power supply. Gel slabs were prepared with 1 % agarose containing 5.4 % ampholine solution (Pharmalyte 3-10, Pharmacia);

0.05 M sulfuric acid and 1 M NaOH were used as anod and cathod buffers, respectively. Proteins were focused at a constant power (0.7 W/cm) for 60 min, with an increasing voltage up to 800 V.

SDS-PAGE (16) was carried out on 10 % polyacrylamide gel slabs, in a Mini-Protean II apparatus (Bio-Rad, Richmond, CA).

Immunostaining of transketolase. Following IEF and SDS-PAGE, proteins were transferred to nitrocellulose by Southern capillary blotting (17) and Western blotting (18), respectively. TK was then detected on NTC with the aid of rabbit anti-TK IgG preparations (see above) followed by biotinilated anti-rabbit antibodies and by the avidin/biotin-immunoperoxidase system (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) as previously reported (13).

Affinity constant determination. Samples (1.5 ml) containing 0.3 u of TK were mixed with polyethylene glycol (7 % final conc.) and centrifuged at 100,000 g for 10 min. The supernatants were incubated for 30 min at 37° C with 300 µl of anti-TK antiserum and spun down as above. Precipitated immunocomplexes, containing about 70 % active TK, were resuspended with 1 ml of 0.1 M tri-ethanolamine-diethanolamine buffer, pH 7.6. This enzyme solution was used for substrate saturation curves. Affinity constants for R5P and Xu5P were calculated by the double reciprocal plots, in the presence of 2 mM cosubstrate concentration.

RESULTS AND DISCUSSION

Total TK content in cell extracts can specifically be detected by a sensitive immunological procedure which does not discriminate between active and inactive forms of the enzyme.

Results in Fig. 1 show a typical IEF analysis of mononuclear leukocyte TK from several healthy donors. All samples yielded heterogeneous profiles consisting of several bands having distinct pI values in a range of 7.4-8.4. These data confirm previous findings on microheterogeneity of TK from rat liver (19), human leukocytes (13) and erythrocytes (9,10), and human and mouse liver (unpublished results). However, the pattern of distribution of immunoreactive material was not the same in all extracts. We could identify a standard TK-profile (S), which is practically identical in the majority of samples (S_{1-6}), and a variant TK-profile ($R_{1,2}$) where enzyme multiple forms are differently arranged. Differences between standard and variant TK are both quantitative -- see the intensity of coincident bands -- and qualitative, like band a which seems characteristically present in the variant. The occurrence of this variant in human population is not as frequent as one might suppose from Fig. 1. In fact, only three unrelated subjects exhibiting the TK-R pattern were found out of 220 donors. So far our results do not confirm the reported abundance of enzyme variants (9) although they definitely prove the occurrence of TK-variants in

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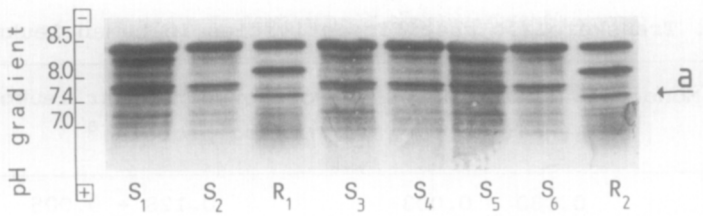

Fig.1. Isoelectrophoretic analysis and detection of transketolase from human mononuclear leukocytes. Cytosolic extracts (20 µl) of mononuclear leukocyte extracts (1-2 mg/ml) were applied to agarose gels, focused and developed as reported in Materials and Methods. Dark bands on nitrocellulose represent reactive material after immunostaining with specific anti-TK IgG preparations. Each standard (S_{1-6}) and variant TK extract (R_{1-2}) was from a different healthy donor. Letter a denotes an enzyme band (pI ca. 7.5) found only in the variant extracts.

human population. Other authors have recently reexamined the isozyme pattern of TK in human erythrocytes (10) concluding that the activity-staining method applied to sixty-three samples did not provide any evidence for enzyme variants. While we share their criticism on the procedure we feel that since TK-variant has a relative frequency of about 1.5 %, it should have been sought over a wider range of subjects to be found.

Differences of IEF pattern between standard and variant TK of mononuclear leukocytes were also evident in pure granulocyte populations (fig. 2), which indicates that the peculiar enzymic profile of variant is maintained in distinct white cell subfamilies. This fact might suggest that multiple TK forms are the products of an individual genetic expression rather than artifacts due to the analysis of mixed cell populations or to

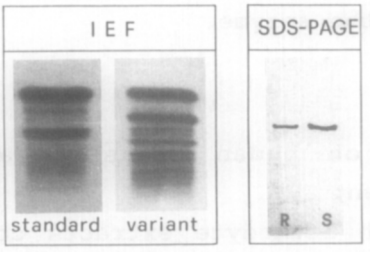

Fig. 2. IEF and SDS-PAGE analysis of transketolase from standard and variant polymorphonuclear neutrophils. Human granulocyte populations (90 % pure) were obtained from whole blood separation on M-PRM. Extraction (see Materials and Methods) and IEF analysis and staining on NTC were as reported in the legend to Fig. 1. Standard (S) and variant (R) granulocyte extracts (10 µl) were also separated on SDS-PAGE under dissociating conditions. Following Western blotting on NTC the enzyme subunits were revealed immunologically as described for the IEF pattern.

TABLE 1. Transketolase specific activities in human leukocytes

	Mononuclear leukocytes	Polymorphonuclear leukocytes
	(unit of TK at 37°C / mg protein)[a]	
STANDARD	0.030 ± 0.003	0.128 ± 0.005
VARIANT	0.023 ± 0.002	0.103 ± 0.005

[a] Specific activities were determined on two separate extracts of both standard and variant subjects and reported ± SD. Leukocyte fractions were separated from blood using M-PRM.

random enzyme degradation during extraction. The variant enzyme from granulocyte extracts has been compared to standard TK using SDS-PAGE and transblotting on NTC. The immunoreactive material is specifically confined to a single band having the same electrophoretic mobility as standard TK. We have also checked the specific activity of TK in standard and variant cell extracts. Values of Table 1 indicate that variant TK has constantly lower specific activities (20-25 % less) than standard TK in both mononuclear and polymorphonuclear leukocyte fraction. The latter is the richest source of TK and it was used for comparative measurements of enzyme affinity constants for substrates. To obtain reliable saturation curves the enzyme was first precipitated from crude extracts with anti-TK antiserum; Km were then determined using isolated active TK-immunocomplex (see Materials and Methods). Under these conditions values obtained with ribose 5-P (0.1 mM) and xylulose 5-P (0.046 mM) were identical for both standard and variant TK and indicated that differences in IEF pattern are not accompanied by changes in the kinetic properties of the enzyme.

CONCLUDING REMARKS

From this study on human transketolase the following conclusions can be drawn:

1) Human TK from fresh leukocyte extracts consists of multiple forms having distinct pI values. Enzyme multiple forms, rather than artifacts, appear to be physiological products and are equally expressed in distinct leukocyte subfamilies.

2) At least two different variants are present in human leukocytes: a major variant (TK-S) represented over 98.5 % of the subjects and a minor variant (TK-R) which accounts for about 1.5 %

of samples examined. A relative frequency of 1 % is sufficient to postulate TK polymorphysm.

3) Apart from differences in IEF patterns and enzyme specific activities of crude extracts, standard and variant TK has similar subunit molecular weight and kinetic constants for ribose 5-P and xylulose 5-P.

4) A sister of a TK-R subject has a standard enzyme profile.

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