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DETERMINATION OF THE SUBUNIT MOLECULAR WEIGHT OF HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE FROM HUMAN ERYTHROCYTES BY RECOVERY OF ENZYME ACTIVITY FROM SODIUM DODECYL SULPHATE GELS

MICHAEL STRAUSS

Academy of Sciences of the German Democratic Republic, Central Institute of Molecular Biology, Division of Bioregulation, Department of Cell Genetics, Berlin-Buch (Germany)

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Summary

The molecular weights of the subunits of the enzyme hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) from human erythrocytes were determined with a simple novel method, including electrophoresis in sodium dodecyl sulphate gels, gel slicing, elution of protein from the gel slices and enzyme reactivation in the presence of the substrate 5-phosphorylribose-1-pyrophosphate. As molecular weight standards glutaraldehyde-polymerized polypeptides of human haemoglobin were used. The experiments clearly showed the existence of molecular weight differences in human erythrocyte hypoxanthine-guanine phosphoribosyltransferase.

In mammals, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) catalyses the conversion of hypoxanthine and guanine to the corresponding mononucleotides by reaction with *P*-Rib-*P*-*P**, thus serving to re-utilize the degradation products of nucleic acids [1].

This enzyme has been the subject of intensive genetic studies in the past [2]. Knowledge obtained from various organisms regarding the structure of hypoxanthine-guanine phosphoribosyltransferase indicates that the character of the enzyme is very heterogenous [3—10], but does not so far permit the unification of all available data to form an integrated idea of the structure.

One of the most important steps in determining the structure is doubtless the precise determination of the number and molecular weights of the individual subunits or polypeptide chains of which the enzyme is comprised.

Two fundamentally different ideas have prevailed in the past. Whereas

Abbreviation: *P*-Rib-*P*-*P*: 5-phosphorylribose-1-pyrophosphate.

*The Mg^{2+} salt of *P*-Rib-*P*-*P* from PL-Biochemicals, Milwaukee, Wisc., U.S.A. was used.

Arnold and Kelley [4] assume a dimeric enzyme (M_r = 68 000) with a subunit of M_r = 34 500, Olsen and Milman [10] propose an enzyme with a subunit of M_r = 26 000. The former authors found, after electrophoresis of the purified enzyme in sodium dodecyl sulphate gels, only one stainable band which corresponded to the assumed molecular weight.

Olsen and Milman, on the other hand, were able to resolve the purified enzyme into at least four stainable bands by sodium dodecyl sulphate gel electrophoresis. One of these bands (M_r = 26 000) was, on the basis of plausible conclusions, regarded as the subunit of hypoxanthine-guanine phosphoribosyltransferase. Since, in connection with genetic investigations, it is necessary to know with absolute certainty whether this enzyme really consists only of polypeptide chains with a uniform molecular weight, we have tried to reactivate the enzyme, after first reducing it, denaturing it and then splitting it in sodium dodecyl sulphate gel in order to obtain a correlation between enzyme activity and molecular weight.

The enzyme used in our studies was isolated and purified by a modified version (Strauss, M., in preparation) of the method employed by Rubin et al. [6]. The two main activity peaks from the anion exchanger column found by Rubin et al. and in our experiment were run separately in sodium dodecyl sulphate gels, three different systems being used:

(1) the continuous buffer system with 10% acrylamide as described by Weber and Osborne [11].

(2) the same system as in (1) with sodium dodecyl sulphate only in the sample solution (0.3%) according to Stoklosa and Latz [12].

And

(3) the discontinuous buffer system described by Davies [13] with 11.1% acrylamide as suggested by Capecchi et al. [14].

The molecular weight standards used were bovine serum albumin, ovalbumin, chymotrypsinogen A and lysozyme on the one hand, and peptide chains from human haemoglobin polymerised by glutaraldehyde as described by Payne [15] on the other.

The last mentioned standards have the advantage of greater linearity of the calibration curve over a broad range. Six clearly distinct bands corresponding to the monomeric and up to the hexameric polypeptides were obtained.

The results obtained from systems 1 and 3 were uniformly good, although the bands obtained from system 3 are rather more distinct and the running time is shorter. System 2 has the advantage that considerably more enzymatic activity can be restored from the gel and the background activity is lower than in the other two systems as a result of the low sodium dodecyl sulphate concentration in the gel discs. Since, however, additional intermediate bands appeared in system 2, which could be due to differences in charging with dodecyl sulphate (see ref. 16).the results obtained from this system cannot be regarded as representative for the actual composition.

The results obtained by means of system 3 are shown in Fig. 1. In order to obtain only subunits, it was absolutely necessary to apply the reducing agent (3—5% 2-mercaptoethanol) before denaturation with sodium dodecyl sulphate. The addition of 1 mM *P*-Rib-*P*-*P* to the elution buffer was absolutely necessary for the partial reactivation of the enzyme.

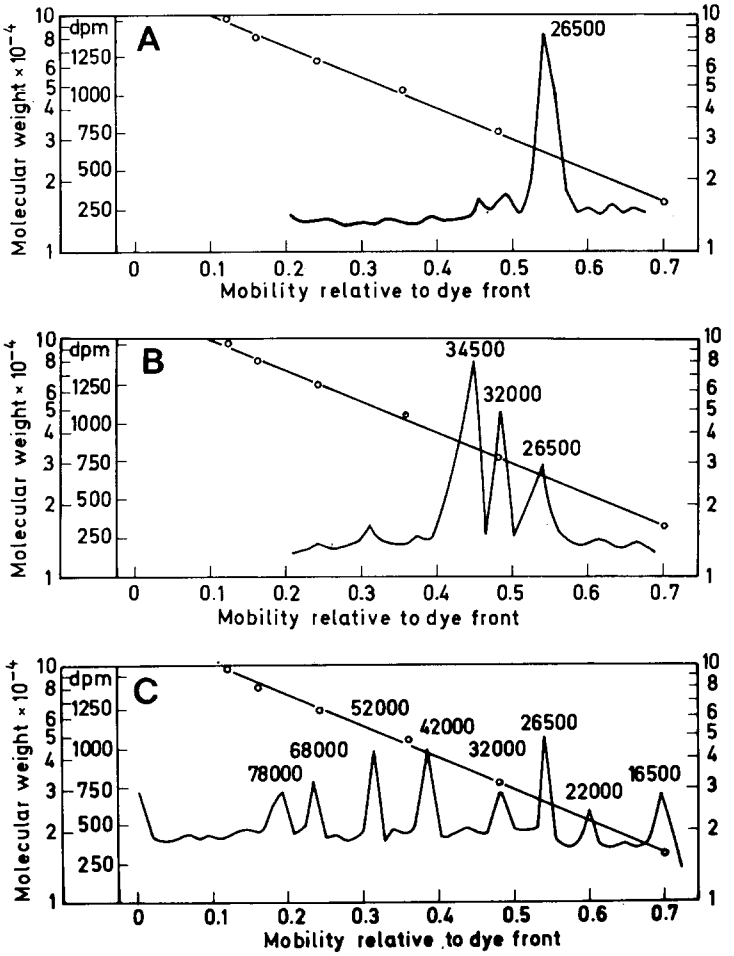

Fig. 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of hypoxanthine-guanine phosphoribosyltransferase and glutaraldehyde-polymerised polypeptide chains of human haemoglobin. Electrophoresis was performed in gel rods, 0.6 x 7 cm, 11.1% acrylamide with the discontinuous buffer system described by Davies [13] including 0.1% sodium dodecyl sulphate and 0.1%-mercaptoethanol. Molecular weight standards were prepared by treatment of 120 µl haemoglobin solution (32 mg/ml) with 10 µl of a glutaraldehyde solution (1%) according to Payne [15]. After incubation at room temperature for 44 h in a stoppered vial, the haemoglobin was diluted to 10 mg/ml, dialysed against 0.02 M Tris-HCl (pH 7.4), reduced with 3% 2-mercaptoethanol and denatured overnight by the edition of 1.5% solid sodium dodecyl sulphate: 20 µl of (the resultant) solution were run in parallel gels. After the bromphenol blue marker had reached the last 0.5 cm of the gel, the gels were removed from the tubes, frozen and cut into 1 or 2-mm slices. Each slice was incubated in 200 µl 0.25 M Tris-HCl (pH 7.6) containing 1 mM *P*-Rib-*P*-*P* at 4° C for 20 h. The recovered proteins were tested for enzyme activity with 0.14 mM [^{14}C]guanine (0.06 µCi) and 1 mM *P*-Rib-*P*-*P* in a total volume of 100 µl for 1 h at 37° C. 40 µl of the test mixture were applied to filter discs which had previously been impregnated with 0.1 M $La(NO_3)_3$ at pH 5.0 according to the method described by Schlossberg and Hollander [18]. After fixation of the nucleotide by incubation on 0.1 M $La(NO_3)_3$, the filters were washed free of guanine with water, ethanol and acetone. The air-dried filter discs were immersed in 5 ml of a counting fluid containing 5 g PPO and 0.1 g POPOP in 1 liter of toluene. Counting vials were shaken for 10 min and counted in a LKB liquid scintillation counter. The gels were stained for proteins with 0.025% coomasie brilliant blue G-250 in water/methanol/acetic acid according to Weber and Osborne [11]. (A) 90 µl (10—20 µg protein) of the first major peak of anion exchanger column, reduced by 3% 2-mercaptoethanol for 1 h at 4° C and denatured with 1% sodium dodecyl sulphate at room temperature overnight. The molecular weight stated is the mean from a total of 6 separations obtained from 3 preparations (± 4%). (B) 90 µl (15—25 µg protein) of the second major peak of the anion exchanger column, reduced and denatured as in (A). The molecular weights stated are the means from a total of 6 separations obtained from 3 preparations (± 4%). (C) 100 µl of combined first and second major peaks of anion exchanger column, adjusted with solid sodium dodecyl sulphate at 1% and immediately afterwards with 3% 2-mercaptoethanol, after standing overnight at room temperature. The molecular weights stated are the means from 4 separations obtained from 2 preparations (± 6.5%).

As can be seen from Fig. 1A, only one subunit can be identified in the first main peak of the anion exchanger column, and this corresponds to that found by Olsen and Milman (M_r = 26 500 or 26 000). In contrast, three electrophoretic peaks can be distinguished in the second main activity peak of the anion exchanger column (Fig. 1B) and these could also correspond to the stainable bands obtained by Olsen and Milman [10]. It is interesting to note that the band with a molecular weight of 34 500 found by Arnold and Kelley was observed as the main activity peak below those mentioned above. This band was the only one, if denaturation and electrophoresis were performed immediately after collection of the second main peak from the column. The two faster peaks appear and their intensities increase with the time of storage. The relative intensities of the three peaks varied from one experiment to the other.

If the protein from the second main column fraction containing the three electrophoretic variants was heated for 10 min at 85°C in the presence of 1 mM *P*-Rib-*P*-*P*, then only the activity peaks with M_r = 26 500 could be found, with nearly unchanged intensity. The intensities of the other two peaks were reduced significantly or were, in some cases, undetectable. This result confirms the findings of Olsen and Milman [10] and suggests that only the enzyme with a subunit having M_r = 26 500 is very stable at 85°C.

Fig. 1C shows the activity pattern obtained when sodium dodecyl sulphate was applied prior to 2-mercaptoethanol. It can be seen clearly that dimeric and trimeric polymers are also separated under these circumstances. Similar stability of oligomers after treatment with sodium dodecyl sulphate and 2-mercaptoethanol has been reported, for example, for human serum albumin [17]. The activity peak with M_r = 16 500 could be found in only two of four experiments. But experiments with chaotropic agents and sulphydryl agents in connection with gel filtration have shown also the existence of two smaller polypeptides with M_r = 20 000. For detection of enzyme activity in these fractions, reassociation to greater units seems to be necessary (unpublished results). Thus, it is possible that the subunits with M_r = 34 500, 32 000 and 26 500 are dimers, but they are obviously the smallest subunits exhibiting enzymatic activity.

The results obtained by the technique described here corroborate those published by other authors regarding the heterogeneous chromatographic and electrophoretic enzyme pattern of hypoxanthine-guanine phosphoribosyltransferase from human erythrocytes [3—8, 10]. However, in contrast to the results obtained by Olsen and Milman [10] and Arnold and Kelley [4], they prove in addition that differences in molecular weight may also be encountered.

In gel filtration experiments the dimeric and trimeric enzymes of all the three subunit types could be found (Strauss, M., in preparation). It seems unlikely that the 2nd main peak of the anion exchanger column represents an enzyme with subunits of different molecular weights. The results rather suggest that the enzyme with subunits of M_r = 26 500 originates from that with a subunit M_r = 34 500 via selective partial degradation, in vitro and possibly in vivo, with the subunit having M_r = 32 000 as an intermediate stage. The subunit with M_r = 34 500 seems to be the primary genetically coded unit.

In our opinion, the results published by Arnold and Kelley [4] and those of Olsen and Milman [10] cannot be regarded as contradictory. Whereas the former authors found the primary enzyme only, the latter found only the secondary enzyme type, as we conclude from their gel filtration experiments and their interpretation of the sodium dodecyl sulphate gel electrophoresis data. However, the electrophoresis data of the latter authors seem to be similar to ours.

The causes for the different results may be due to differences in the material used.

From 9 preparations under identical conditions (Strauss, M., in preparation) we found in five cases native enzymes corresponding to the assumed primary and secondary subunit types and in four cases a native enzyme with secondary subunits only. In no case did we find the primary enzyme type alone.

We cannot yet say whether the different degree of conversion correlates with the metabolic situation or the composition of the erythrocyte population in vivo or whether the degradation occurs in vitro only. The possible participation of proteolysis in the conversion process is under investigation.

In our gel filtration experiments we could find up to nine activity peaks representing various oligomers and degradation products, but recent results suggest that obviously only one or two active forms of hypoxanthine-guanine phosphoribosyltransferase are present in human red cells in vivo (Strauss, M., in preparation).

With the technique described in this paper it was possible to overcome the difficulties in coordination of protein staining with enzyme activity after denaturation with sodium dodecyl sulphate. After dilution of sodium dodecyl sulphate, renaturation of an active enzyme takes place in the presence of the substrate *P*-Rib-*P*-*P*. This technique also permits estimation of molecular weights in relatively crude enzyme preparations.

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