Yeast Phosphoglycerate Mutase

CYANOGEN BROMIDE CLEAVAGE AND AMINO ACID SEQUENCE OF AN ACTIVE-SITE PEPTIDE

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(Received 10 July 1975)

The molecular weight and amino acid composition of phosphoglycerate mutase from yeast were determined. CNBr cleavage produced a large (190-residue) fragment and a small (60-residue) fragment. Tryptic and chymotryptic peptides derived from the large fragment were fractionated by ion-exchange chromatography. Peptides from two histidine-containing regions were isolated and the amino acid sequences determined. Correlation of these data with X-ray-crystallographic evidence shows that the histidine residue in the sequence Arg-Leu-Asn-Glu-Arg-His-Tyr-Gly-Asp-Leu-Glu-Gly-Lys is located at the active site. Evidence of homogeneity of the protein, fractionation of CNBr fragments and details of the method of peptide separation have been deposited as Supplementary Publication SUP 50060 (11 pages) at the British Library (Lending Division), Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, U.K., from whom copies may be obtained on the terms indicated in *Biochem. J.* (1976), 153, 5.

The structure of yeast phosphoglycerate mutase (EC 5.4.2.1) has been determined to 0.35 nm (3.5 Å) resolution (Campbell *et al.*, 1974). It is a tetrameric enzyme with identical subunits, and shows many similarities to certain nucleotide-binding enzymes (Adams *et al.*, 1970; Rossman *et al.*, 1971; Hill *et al.*, 1972; Buehner *et al.*, 1973; Brändén *et al.*, 1973; Bryant *et al.*, 1974; Blake & Evans, 1974). Phosphoglycerate mutase, however, does not require nucleotide binding for activity, and more detailed examination of its structure may provide information about its evolution and function.

Phosphoglycerate mutase catalyses the inter-conversion of 3- and 2-phosphoglyceric acid (Meyerhof & Kiessling, 1935), and requires 2,3-diphosphoglyceric acid as coenzyme (Grisolia & Joyce, 1959). Active-site studies have shown the formation of an intermediate with a phosphorylated histidine residue (Rose, 1970), and that amino groups are also important for activity (Sasaki *et al.*, 1971).

We have isolated and sequenced the two histidine-containing regions derived from a 190-residue CNBr fragment from the *N*-terminus of the enzyme. X-ray-diffraction evidence had indicated that the active site was contained within this fragment. The amino acid sequence of one of the histidine regions correlates well with the 0.35 nm electron-density map in the vicinity of the proposed active site. The other histidine residue appears to be located in a different portion of the molecule.

Experimental and Results

Materials

Chymotrypsin and trypsin were from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Subtilisin BPN' and insulin were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Phenyl isothiocyanate and trifluoroacetic acid were Sequanal grade from Pierce Chemical Co., Rockford, Ill., U.S.A. Dansyl chloride, polyamide plates and dimethylaminobenzaldehyde were from BDH Chemicals, Poole, Dorset, U.K.

Purification of phosphoglycerate mutase

Phosphoglycerate mutase was prepared from dried baker's yeast (*Saccharomyces cerevisiae*) supplied by Distillers Co. as described by Morena *et al.* (1968), except that lysis time was shortened to 9 h, and that Fraction 2 was dialysed before acetone precipitation at 0°C. Additional purification incorporated gel filtration (Campbell *et al.*, 1971) and several recrystallizations. The enzyme was homogeneous, as indicated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber *et al.*, 1972). Details are given in Supplementary Publication SUP 50060.

Molecular-weight determination

The molecular weight of native phosphoglycerate mutase was determined by the short-column

equilibrium-sedimentation method (Chervenka, 1969), by using a Beckman model E ultracentrifuge. Centrifugation was at 12500 rev./min for 47 h in an An-D rotor, with a column height of 2.2 mm and a protein concentration of 5 mg/ml. The partial specific volume was assumed to be 0.73 cm³/g (e.g. Tanford, 1961). The value obtained for the tetramer was 112400, giving a monomer mol.wt. of 28100.

The monomer molecular weight estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber *et al.*, 1972) was 27500.

The molecular weight of the CNBr fragments was estimated by calibration of the Sephadex column used in their purification. The large fragment (CNBr 1) was eluted in the void volume (mol.wt. >15000), the small fragment (CNBr 2) had mol.wt. approx. 5500.

Cyanogen bromide cleavage

The mutase (48 mg) was dissolved in 5 ml of 70% (v/v) formic acid, and approx. 50 mg of CNBr was added. The mixture was left at room temperature (18–24°C) for 24 h and then applied to a Sephadex G-50 column (2 cm × 60 cm) and eluted with 50% (v/v) formic acid. The elution profile is shown in Supplementary Publication SUP 50060. Two fragments were obtained corresponding to mol.wt. >15000 (CNBr 1) and mol.wt. approx. 5500 (CNBr 2).

Amino acid analysis

Phosphoglycerate mutase and CNBr fragments were hydrolysed in evacuated tubes with 0.5 ml of 5.5 M-HCl at 110°C for 20, 48 or 96 h. Peptide samples were hydrolysed for 20 h. Quantitative analyses were performed on a single column (0.3 cm × 100 cm), eluted by an 8 h cycle of stepwise buffer changes. Tryptophan was determined colorimetrically by the method of Spies & Chambers (1948). The results are presented in Table 1.

Isolation of possible active-site peptides

Evidence from the X-ray-diffraction data indicated that the active site was in the *N*-terminal three-quarters of the molecule (Campbell *et al.*, 1974). Amino acid analysis showed the molecule had only one methionine residue. Fragment CNBr 1 was selected for further study since it contained homoserine, and was therefore derived from the *N*-terminus.

Fragment CNBr 1 was pooled from three filtration experiments and freeze-dried. The fragment (approx. 75 mg or 4 μmol) was dissolved in 10 ml of 1% (w/v) NH₄HCO₃, pH 7.9. Trypsin or chymotrypsin was added to give a final enzyme/substrate ratio of 1:50 (w/w). Digestion was at 37°C for 17 h,

Table 1. *Amino acid composition of phosphoglycerate mutase and its CNBr fragments*

Experimental details are described in the text. Values of serine and threonine are corrected by extrapolation to zero time. Other values are averages from the three different times of hydrolysis.

Amino acid	Composition (residues/molecule)			
	Phosphoglycerate mutase	CNBr 1	CNBr 2	CNBr 1 + CNBr 2
Asx	29.3	20.9	6.7	27.6
Thr	8.7	8.2	1.1	9.3
Ser	15.8	13.2	4.1	17.3
Glx	28.8	23.5	5.0	28.5
Pro	16.7	12.7	4.6	17.3
Gly	16.4	10.9	5.4	16.3
Ala	24.0	14.0	10.0	24.0
Cys	0.0	0.0	0.0	0.0
Val	15.5	12.6	2.5	15.1
Met	1.0	0.0	0.0	0.0
Ile	10.0	6.0	3.9	9.9
Leu	26.7	19.7	6.4	26.1
Tyr	8.5	7.0	1.6	8.6
Phe	5.9	5.3	0.9	6.2
Lys	24.4	18.7	5.2	23.9
His	3.9	2.2	1.7	3.9
Arg	12.0	11.5	1.0	12.5
Hse	—	+	—	+
Trp	4.4	4.9	0.0	4.9
Total residues	254	194	61	255
Mol.wt.	28317	22078	6367	28535

and was stopped by freeze-drying. The peptides were applied to a sulphonic cation-exchange resin and were eluted by a gradient of pyridine and acetic acid. Details of the separation, and elution profiles of tryptic and chymotryptic digests, are shown in Supplementary Publication SUP 50060.

After separation of chymotryptic peptides the resin was removed from the column and washed with two 25 ml batches of conc. NH₃ (sp.gr. 0.88)/pyridine (1:1, v/v). Four additional peptides were obtained. One of these peptides (CAPc) gave a positive reaction with Pauly reagent (Offord, 1969) indicating the presence of histidine or tyrosine, and was shown to contain histidine and tyrosine by amino acid analysis.

Further purification of peptides was carried out when necessary by high-voltage electrophoresis at pH 6.5 or 2 (Fothergill & Fothergill, 1970). At pH 6.5, peptide mobility was expressed relative to aspartic acid, with valine as a neutral marker. At pH 2 mobility was measured relative to valine with dansyl-OH (5-dimethylaminonaphthalene-1-sulphonic acid) as a neutral marker.

Peptide nomenclature

Upper-case letters indicate that the peptide was derived from digestion by: C, chymotrypsin; S, subtilisin; T, trypsin. Numerals immediately after the 'C' or 'T' refer to the peak number from the ion-exchange fractionation, and 'AP' is a peptide from the ammonia/pyridine wash. Lower-case letters refer to the electrophoretic mobility at pH6.5, with 'a' being the most acidic. Numerals refer to electrophoretic mobility at pH2 with '1' being the most acidic.

Amino acid-sequence determination

The amino acid sequences were determined by using the dansyl-Edman method as described by Hartley (1970), except that 2% (v/v) triethylamine was incorporated in the 50% (v/v) pyridine. Dansyl-amino acids were identified by two-dimensional t.l.c. (Woods & Wang, 1967) on polyamide plates (5cm×5cm). Dns-Arg, Dns-His and ε-Dns-Lys were separated by ethanol/0.05M- Na_3PO_4 (1:3, v/v) (Hartley, 1970) after the first three solvents. Subtractive Edman determinations were also performed, and agreed with the dansyl identifications.

The presence of tryptophan was indicated by reaction with Ehrlich reagent (Smith, 1953).

For peptide T20a it was necessary to carry out sub-digestions with chymotrypsin and subtilisin. Approx. 250nmol of peptide T20a was dissolved in 1ml of 1% (w/v) NH_4HCO_3 , pH7.9, and approx. 125 μg of enzyme was added. Digestion was for 1–2h at 37°C.

The amide content of a peptide was determined by correlating its electrophoretic mobility at pH6.5 and its molecular weight (Offord, 1966). If the amide position was still ambiguous, the electrophoretic mobility of the peptide after the appropriate number of rounds of Edman degradation was determined.

The sequences of the two histidine-containing regions from CNBr 1 are shown in Fig. 1. The amino acid compositions and electrophoretic mobilities of these peptides are presented in Table 2.

The mobility at pH6.5 of peptide CAPc from Region 1 indicated that it has one acidic side chain and one amide group. Moreover, peptide T15.3 is neutral at pH6.5. The amide group is located at position 3, since the mobility at pH6.5 of peptide CAPc was +0.22 after two rounds of Edman degradation, and +0.23 after three rounds. The mobilities of peptide T15.3 after one and two

Fig. 1. Amino acid sequences of the two histidine-containing regions from fragment CNBr 1

Residues were identified by dansyl-Edman degradation (→). Peptide nomenclature is described in the text.

Table 2. *Electrophoretic mobilities and amino acid compositions of peptides from histidine-containing regions*
Experimental details and peptide nomenclature are given in the text. Composition values are uncorrected.

Peptide	Mobility		Composition (mol/mol of peptide)											Total
	pH6.5	pH2	Asx	Ser	Glx	Gly	Val	Leu	Tyr	Lys	His	Arg	Trp	
CAPc	+0.39		1.2		1.0			1.0	0.6		0.9	1.8		7
T15.3	0	+1.25	1.2		1.1			0.6				0.8		4
T19	+0.06	+1.27	1.1		1.2	1.9		1.0	1.0	1.0	1.0			8
C45	+0.17	+1.17		1.1	2.1	1.1	1.0				1.0	0.9	+	8
T20a	-0.19	+1.17	1.1	1.0	3.1	1.0				0.9	0.7		+	9
T20aCa	-0.25			0.9	1.9	1.0					0.4		+	6
T20aCc	0		1.0		1.2					0.9				3
T20aSbc2	0	+1.13	1.1		1.0					0.9			+	4
T20aSa	-0.39			1.0	1.9	1.1							+	5
C45Sa	-0.56				0.9								+	2
C45Sb	-0.50			0.8	1.2								+	3
C45Sc	-0.44			1.0	2.0								+	4

Fig. 2. *Correlation of amino acid sequence with electron density*

The sequence Arg-His-Tyr is shown superimposed on the 0.35 nm (3.5 Å) electron-density map (Campbell *et al.*, 1974).

rounds of degradation substantiated this result. Residues 9 and 11 are acidic, since peptide T19 is neutral at pH6.5.

Region 2 has two acidic side chains and two amide groups from the mobility at pH6.5 of peptide T20a. The glutamine at position 5 and glutamic acid at 6 were assigned from the acidic mobilities of peptides C45Sa and C45Sb. The glutamine at position 9 and aspartic acid at 10 were deduced from the mobility at pH6.5 of peptide T20aSbc2 being neutral after two rounds of Edman degradation.

Discussion

The molecular weight of the phosphoglycerate mutase reported here agrees well with previously measured values. Edelhoch *et al.* (1957) found a tetramer mol.wt. of 112000; Rose (1971), using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, determined a subunit mol.wt. of 27000. The amino acid composition was essentially the same as that reported by Sugimoto *et al.* (1963).

The sequence determination of Region 1 (see

Fig. 1) presented no difficulties except that the chymotryptic peptide CAPc was bound very firmly to the ion-exchange resin, and was not found until its absence from the elution profile had been established. The sequence determination of Region 2 sometimes did not progress beyond the glutamine residues, and may have been due to formation of pyrrolidonecarboxylic acid.

A 0.35 nm (3.5 Å) resolution electron-density map of phosphoglycerate mutase has been calculated (Campbell *et al.*, 1974), which shows that the four subunits are arranged with almost perfect 222 symmetry to form a compact molecule whose shape is best described as a flattened sphere. In the absence of sequence information the electron density associated with each subunit has been interpreted in terms of 220 amino acid residues.

The somewhat tentative positioning of the active-centre region (see Campbell *et al.*, 1974) has now received support from additional X-ray-crystallographic experiments using crystals grown after reaction with trinitrobenzenesulphonic acid (Sasaki *et al.*, 1971). Use of this reagent leads to 75% loss of enzymic activity and produced a positive peak in the resulting electron-density map in the region of the 3-phosphoglycerate-binding site.

The sequences of the two histidine-containing regions found in the large CNBr fragment have been compared with the indications of side chains in the electron-density map. Each region correlates well with a unique portion of the electron-density map. Fig. 2 illustrates the excellent agreement found between the amino acid sequence about the histidine in Region 1 and the side-chain density in the vicinity of the 3-phosphoglycerate-binding site. We believe that this result confirms the location of the active-centre region, and clearly points to the histidine residue in Region 1 as the one implicated by Rose (1970) as forming a phosphoryl intermediate during catalysis.

The histidine residue in Region 2 appears to be located at a distant portion of the molecule, although the lysine residue at its C-terminus approaches the active site again, and may be implicated in the trinitrobenzenesulphonic acid inactivation of the mutase.

We thank Dr. R. P. Ambler, Dr. J. E. Fothergill, Dr. H. C. Watson and Dr. S. P. Spragg for helpful discussions, Mrs. Jean Bathgate for skilful technical assistance, and the M.R.C. for financial support.

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