Glucose Phosphorylation

INTERACTION OF A 50-AMINO ACID PEPTIDE OF YEAST HEXOKINASE WITH TRINITROPHENYL ATP *

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A 50-amino acid peptide predicted by chemical modification studies of yeast hexokinase to contain an ATP-binding site has been synthesized and purified. The peptide, which includes residues from glutamate 78 at the NH₂-terminal end to leucine 127 at the COOH-terminal, resides within the smaller of the two lobes found in the three-dimensional structure of yeast hexokinase. It is this region which has been reported recently to exhibit significant sequence homology with hexokinase types I and IV of higher eukaryotic cells and sequence homology with the active site of protein kinases.

Similar to native yeast hexokinase, the 50-amino acid peptide interacts strongly with the fluorescent analog TNP-ATP [2',3'-O-(2,4,6-trinitrophenyl)-adenosine-5'-triphosphate]. A 5-fold enhancement is observed when 8 μM peptide interacts with 20 μM TNP-ATP. The stoichiometry of binding is very close to 1 mol of TNP-ATP/mol peptide. Also, similar to native yeast hexokinase, the fluorescent enhancement observed upon TNP-ATP binding to the synthetic peptide is greater than that observed upon TNP-ADP binding. Finally, TNP-AMP exhibits a much lower fluorescent enhancement in the presence of hexokinase or the synthetic peptide.

The additional findings that ATP can readily prevent TNP-ATP binding and that TNP-ATP can substitute for ATP as a weak substrate for hexokinase in the phosphorylation of glucose indicate that the synthetic peptide described here comprises part of the catalytic site.

Hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) commits glucose to catabolism by catalyzing the phosphorylation of glucose using ATP in the presence of Mg²⁺. Only the three-dimensional structure of the yeast enzyme has been elucidated (1–6). As shown in Fig. 1A, the enzyme consists of a single polypeptide chain of 50 kDa which folds into a three-dimensional structure in which there is a deep central cleft that divides two lobes (1–4). Upon binding glucose, the smaller lobe rotates relative to the larger lobe partially closing the cleft around the glucose molecule (4).

The region of the hexokinase molecule involved in ATP binding remains controversial. Although the x-ray studies indicate that the ATP-binding site lies within the larger of the two lobes, it is important to note that 5-BrAMP rather than ATP was used (6). Recent work from other laboratories now indicates that the major region of ATP binding to yeast hexokinase may lie within the smaller lobe. Thus, Tamura et al. (7) demonstrated that the ATP affinity label PLP-AMP¹ (pyridoxyl 5'-diphosto-5'-adenosine) binds covalently to lysine 111 within the smaller lobe. Also, amino acid sequence analysis of several eukaryotic hexokinases, including types I and IV, and yeast types A and B have revealed considerable sequence homology within the smaller lobe (8–12). Part of this region matches closely the ATP binding region found in protein kinases (13–16).

In order to test more directly the role of the smaller lobe of yeast hexokinase in ATP binding, we have synthesized a 50-amino acid peptide which includes residues from glutamate 78 at the NH₂-terminal to leucine 127 at the COOH-terminal (Fig. 1B). This peptide includes both the region of homology found in various hexokinase forms and lysine 111 which binds the ATP affinity label (7). Data presented below demonstrate that this synthetic peptide retains significant structure and binds the fluorescent ATP analog TNP-ATP.

EXPERIMENTAL PROCEDURES

Materials

All the Boc-protected amino acids, Boc-Leu-OCH₃-PAM resin, and the reagents and solvents for the peptide assembly were purchased from Applied Biosystems, Inc. Trifluoroacetic acid, p-cresol, p-thiocresol, and dimethyl sulfoxide were supplied by Aldrich. Hydrogen fluoride was from Matheson Gas Products, Inc. Acetonitrile, methylene chloride, and dimethylformamide (all HPLC grade) were obtained from Baxter Health Care Corporation. Amino acid standards and phenylisothiocyanate were obtained from Pierce Chemical Co. All reagents and phenylthiohydantoin-amino acid standards for NH₂-terminal sequencing of the peptide were procured from Applied Biosystems, Inc. ATP was obtained from Pharmacia LKB Biotechnology, Inc. Yeast hexokinase Type C-300 (monomeric molecular weight = 60,000) was purchased from Sigma. MgCl₂, Tris Cl, and Dowex 2X-100 were purchased also from Sigma, whereas D-glucose was obtained from J. T. Baker Chemical Co. [U-¹⁴C]Glucose was from Amersham Corp. TNP-nucleotides were purchased from Molecular Probes, and their purities confirmed by chromatography on polyethyleneimine-cellulose plates (Cel-300 PEI, Brinkmann Instruments) in a solvent

¹ The abbreviations used are: PLP-AMP, pyridoxyl 5'-diphosto-5'-adenosine; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate; TNP-ADP, 2',3'-O-(2,4,6-trinitrophenyl)-adenosine 5'-diphosphate; TNP-AMP, 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-monophosphate; HPLC, high pressure liquid chromatography, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPP-50, synthetic peptide comprising glutamate 78 through leucine 127 of yeast hexokinase.

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FIG. 1. A, computer graphics representation of yeast hexokinase depicting the two lobes, large and small, enclosing a central cleft where glucose binds (3). The synthetic peptide employed in this study called HPP-50 includes residues from glutamate 78 at the NH2-terminal end to leucine 127 at the COOH-terminal and is located within the small lobe as indicated in pink. The X shown in yellow represents the position of the basic amino acid residue to which an ATP photoaffinity label binds (7). The graphics were obtained with an Evans Sutherland PS 390 system with the use of the program FRODO (30). The x-ray coordinates for yeast hexokinase were obtained from the Brookhaven Protein Data Bank. B, the amino acid sequence of HPP-50 and homologous regions of other proteins (10). Amino acid residues enclosed by boxes are conserved in yeast hexokinase types A and B within the region of HPP-50, in hexokinase types I (brain and kidney) and IV (liver glucokinase), and in several protein kinases (13-16).
was obtained using an AVIV 60DS spectrometer. Spectra were recorded at 25 °C using a Hellma rectangular demountable cuvette facility.

Amino acid sequencing was performed on an Applied Biosystems model 470A protein sequencer in the University Protein/Peptide dissolving in distilled water or 10 mM HCl. The reaction mixture contained in a final volume of 1.0 ml, 10 mM Tris-Cl, pH 7.4, 50 μg HPP-50 in 6 N HCl + 1% (v/v) phenol at 110 °C for 24 h. Amino acid sequencing was performed on an Applied Biosystems model 470A protein sequencer in the University Protein/Peptide facility.

Circular Dichroism of HPP-50—The circular dichroism spectra were obtained using an AVIV 60DS spectrometer. Spectra were recorded at 25 °C using a Hellma rectangular demountable cuvette made of Suprasil quartz with a 0.1-mm path length.

Quantification of HPP-50—HPP-50 was quantified by the bicinchoninic acid protein assay (Pierce Chemical Co.) using bovine serum albumin as standard. This procedure gave values for HPP-50 within 8% of those obtained by direct weighing. HPP-50 was routinely dissolved in distilled water or 10 mM Tris-Cl buffer, pH 7.4, at concentrations as high as 1 mg/ml without any problems from aggregation or precipitation.

Interaction of HPP-50 with TNP-Nucleotides—This was monitored fluorometrically in a 4-ml quartz cuvette (Starna Suprasil) containing in a total volume of 2 ml, 10 mM Tris-Cl, pH 7.4, and concentrations of TNP-nucleotides, HPP-50, glucose, and MgCl2 as indicated in legends to the figures. An Aminco SPF-125° spectrophotometer set at an excitation wavelength of 405 nm and an emission wavelength of 520 nm was used for these studies.

Determination of Stoichiometries and Dissociation Constants—Stoichiometries (i.e. mol TNP nucleotide/mol HPP-50 or per mol hexokinase) were estimated by extrapolating from the initial part of the titration curves (where it is assumed that all substrate is bound) to the maximal fluorescent change. Dissociation constants apparent (Kd values) were estimated by the graphical method of Stimson and Holbrook (19). The molecular weights of HPP-50 and monomeric yeast hexokinase used were, respectively, 6,210 and 50,000.

Circular dichroism of HPP-50 in SDS—SDS-PAGE was carried out by a modification of the Weber and Osborn procedure (20) in 10% polyacrylamide gels exactly as described previously (21).

Results and Discussion

Purity and Characterization of the 50-Amino Acid Residue Hexokinase Peptide (HPP-50)—Procedures employed in the synthesis and purification of HPP-50 are described in detail under "Experimental Procedures." As shown in Fig. 2A and Table I, four different methods were used to examine the purity of HPP-50 prior to investigating its capacity to interact with ATP. Fig. 2A shows that upon HPLC chromatography on a μBondapak C8 column, HPP-50 elutes as a single peak, nearly gaussian in shape. The inset in Fig. 2A shows that HPP-50 migrates as a single band upon SDS-PAGE in a 10% polyacrylamide gel system. As predicted from its molecular weight of 6,210, HPP-50 migrates faster than cytochrome c (M, = 11,700). HPP-50 appears by this criteria apparently homogeneous with neither larger nor smaller contaminants being apparent. In data not presented here, HPP-50 also showed a single band upon native PAGE in a 20% polyacrylamide gel. Results presented in Fig. 2B show that the amino acid composition of HPP-50. The amino acid composition containing [U-14C]glucose, 0.2 × 106 cpm/μmol, and 100 μg of yeast hexokinase added in 30 μl of 10 mM Tris-Cl buffer, pH 7.4. The reaction was initiated by addition of 1 mM TNP-ATP and terminated at appropriate times by the addition of 4 ml, 1 mM glucose in 0.17 M NH4OH. Labeled glucose 6-phosphate was then separated from other components of the reaction mixture by ion exchange chromatography using untreated, dry Dowex 2X8-100 (50-100 mesh) resin. This resin (0.7-0.9 g) was added directly to the reaction mixture, vigorously agitated for 6 min, and then sedimented in a bench top centrifuge. The resin was washed six times with 4 ml of 0.17 M NH4OH, treated with 1 ml, 1 M HCl and agitated for 5 min at 2 min intervals. A 0.2-ml aliquot of the acidic supernatant containing the labeled glucose 6-phosphate was assayed for radioactivity in 10 ml of "Budget Solve" in a Beckman LS-100C liquid scintillation counter.

Amino Acid Analysis and Sequencing—Amino acid analysis on phenylthiocarbamyl derivatives (18) was performed by the PICO-PAIR amino acid analysis system of Waters-Millipore after hydrolyzing 50 μg HPP-50 in 6 N HCl + 1% (v/v) phenol at 110 °C for 24 h. Amino acid sequencing was performed on an Applied Biosystems model 470A protein sequencer in the University Protein/Peptide facility.

Trifluoroacetic acid. HPP-50 in the eluate was detected at 220 nm with 1 ml, 1 M HCl. The fractions were analyzed, and those containing only the pure peptide were pooled and freeze dried. Trailing and leading edges were discarded. The yield of pure peptide was 17.5 mg (10%). This yield, although somewhat low, was accepted at the cost of purity.

Hexokinase Peptide

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Methods

Stoichiometries (i.e. mol TNP nucleotide/mol HPP-50) are summarized in Table I. The titration curves (where it is assumed that all substrate is bound) to the maximal fluorescent change. Dissociation constants apparent (Kd values) were estimated by the graphical method of Stinson and Osborn (20) in a 10% polyacrylamide gel system. As predicted from its molecular weight of 6,210, HPP-50 migrates faster than cytochrome c (Mr = 11,700). HPP-50 appears by this criteria apparently homogeneous with neither larger nor smaller contaminants being apparent. In data not presented here, HPP-50 also showed a single band upon native PAGE in a 20% polyacrylamide gel. Results presented in Fig. 2B show that the amino acid composition of HPP-50. The amino acid composition containing [U-14C]glucose, 0.2 × 106 cpm/μmol, and 100 μg of yeast hexokinase added in 30 μl of 10 mM Tris-Cl buffer, pH 7.4. The reaction was initiated by addition of 1 mM TNP-ATP and terminated at appropriate times by the addition of 4 ml, 1 mM glucose in 0.17 M NH4OH. Labeled glucose 6-phosphate was then separated from other components of the reaction mixture by ion exchange chromatography using untreated, dry Dowex 2X8-100 (50-100 mesh) resin. This resin (0.7-0.9 g) was added directly to the reaction mixture, vigorously agitated for 6 min, and then sedimented in a bench top centrifuge. The resin was washed six times with 4 ml of 0.17 M NH4OH, treated with 1 ml, 1 M HCl and agitated for 5 min at 2 min intervals. A 0.2-ml aliquot of the acidic supernatant containing the labeled glucose 6-phosphate was assayed for radioactivity in 10 ml of "Budget Solve" in a Beckman LS-100C liquid scintillation counter.

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adenine nucleotides, we used the fluorescent probe TNP-ATP. This nucleotide analog represents a sensitive probe for mine whether HPP-50 exhibits the capacity to interact with ATP-binding sites and has been used successfully in probing the active sites of both the mitochondrial (23) and Na+,K+ ATPase (24). Results presented in Fig. 3A show that HPP-50 interacts with TNP-ATP throughout the concentration range of TNP-ATP tested. Overall, the relative degree of interaction is in the order TNP-ATP > TNP-ADP > TNP-AMP. The recoveries of amino acids with reactive side chains like Glu, Asp, Ser, and Thr were low because of their degradation during the trifluoroacetic acid treatment to form phenylthiohydantoin amino acid derivatives. Amino acids with stable side chains like Phe, Gly, Leu, Ala, and Val were recovered in good yields.

| Cycle | Amino acid | Yield* (pmol) |
|-------|------------|--------------|
| 1     | Glu        | 210.6        |
| 2     | Ser        | 287.1        |
| 3     | Gly        | 544.3        |
| 4     | Asp        | 135.0        |
| 5     | Phe        | 551.1        |
| 6     | Leu        | 631.6        |
| 7     | Ala        | 565.2        |
| 8     | Ile        | 457.2        |
| 9     | Asp        | 226.0        |
| 10    | Leu        | 481.1        |
| 11    | Gly        | 335.9        |
| 12    | Gly        | 410.6        |
| 13    | Thr        | 351.0        |
| 14    | Asn        | 199.1        |
| 15    | Leu        | 348.9        |

*Values shown are pmol of yields of the phenylthiohydantoin amino acid derivatives starting with approximately 600 pmol of the purified HPP-50 peptide.

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The amino acid sequence (38 residues) of HPP-50 purifies HPP-50 peptide.

Data presented in Fig. 3A show also that neither Mg** nor glucose are required for TNP-ATP binding to HPP-50. The latter observation is consistent with the x-ray structure that predicts that residues involved in glucose binding lie outside the region of HPP-50 (1). Therefore, the binding of TNP-ATP to HPP-50 cannot experience a dependence on glucose binding as is the case in the intact enzyme (Fig. 4A). Finally, it is important to note in Fig. 3B that the addition of ATP prior to TNP-ATP to HPP-50 almost completely prevents a fluorescent response. In data not presented here, ATP addition after TNP-ATP addition also reversed the fluorescence response. Thus, the site on HPP-50 which interacts with TNP-ATP also appears to interact with ATP.

Interaction of HPP-50 with TNPP-ATP—In order to determine whether HPP-50 exhibits the capacity to interact with adenine nucleotides, we used the fluorescent probe TNP-ATP. This nucleotide analog represents a sensitive probe for ATP-binding sites and has been used successfully in probing the active sites of both the mitochondrial (23) and Na+,K+ ATPase (24). Results presented in Fig. 3A show that HPP-50 induces TNP-ATP to exhibit a strong fluorescent enhancement throughout the concentration range of TNP-ATP tested (≤20 μM). HPP-50 induces a similar response upon interacting with TNP-ADP but a much lower response in the presence of TNP-AMP. Overall, the relative degree of interaction is in the order TNP-ATP ≈ TNP-ADP >> TNP-AMP. Stoichiometric ratios (mean values) for TNP-ATP and TNP-ADP were found to be, respectively, 0.80 and 0.85 mol/mol HPP-50 with apparent dissociation constants ($K_D$ values) of 4.4 and 5.2 μM.

Data presented in Fig. 3A show also that neither Mg** nor glucose are required for TNP-ATP binding to HPP-50. The latter observation is consistent with the x-ray structure that predicts that residues involved in glucose binding lie outside the region of HPP-50 (1). Therefore, the binding of TNP-ATP to HPP-50 cannot experience a dependence on glucose binding as is the case in the intact enzyme (Fig. 4A). Finally, it is important to note in Fig. 3B that the addition of ATP prior to TNP-ATP to HPP-50 almost completely prevents a fluorescent response. In data not presented here, ATP addition after TNP-ATP addition also reversed the fluorescence response. Thus, the site on HPP-50 which interacts with TNP-ATP also appears to interact with ATP.
values) for binding of TNP-ATP and TNP-ADP to the native enzyme were in the same range as those for the synthetic peptide. The stoichiometry for TNP-ATP binding was 0.85 mol/mol hexokinase (50 kDa) in the absence of glucose and 0.6 in the presence of glucose. The apparent $K_D$ value in the presence of glucose was 1.1 $\mu$M and in the absence of glucose 2.6 $\mu$M. For TNP-ADP binding the stoichiometry was 0.75 mol/mol hexokinase with an apparent $K_D$ value of 3.7 $\mu$M. (The apparent dissociation constant for the binding of ATP to yeast hexokinase has been determined by various investigators and lies in the range of 200–2000 $\mu$M (25, 26). Therefore, TNP-ATP binding to hexokinase appears much tighter than that of ATP, a finding consistent with the binding of TNP-ATP to the catalytic sites of the mitochondrial F$_1$-ATPase (23) and the Na$^+$/K$^+$ ATPase (24).

Overall, the data summarized in this report indicate that binding of TNP-ATP to both HPP-50 and to hexokinase is not due to nonspecific hydrophobic interactions, but reflects specific binding. Thus, binding is prevented or reversed by addition of the substrate ATP. Moreover, TNP-AMP binds poorly to both HPP-50 and to hexokinase. In experiments not presented here, it should be noted also that TNP-ATP did not bind to several other proteins tested including lactoglobulin, chymotrypsinogen, and ovalalbumin. Also, TNP-ATP failed to bind to a synthetic, 50 amino acid, yeast hexokinase peptide (tyrosine 322 through isoleucine 371), which showed very little secondary structure.

Significantly, two different methods not presented here revealed that TNP ATP can substitute for ATP in the hexokinase reaction as a weak substrate. Using the thin layer method normally used to assess the purity of TNP-nucleotides (see "Experimental Procedures"), the presence of hexokinase was observed visually to induce the formation of TNP-ADP. In a second assay (see "Experimental Procedures") in which the formation of $[^{14}C]$glucose 6-phosphate from $[^{14}C]$glucose and TNP-ATP was monitored, a very low but significant rate (420 nmol of glu-6-P/h/mg protein) of product formation was observed. (This low rate may reflect tight binding of the product TNP-ADP to hexokinase, its slow release rate impairing further catalysis.)

In summary, the studies presented here add support to the conclusion of Tamura et al. (7) that the smaller lobe of hexokinase is involved in ATP binding. They also support the recent suggestion of Andreone et al. (10) based on homology arguments that the region of yeast hexokinase (and other hexokinases), comprised here by the synthetic peptide HPP-50, includes an ATP-binding site similar to that found also in protein kinases. However, unlike putative ATP binding domains reported for adenylate kinase (27), the mitochondrial ATP synthase (28), and many other nucleotide-binding proteins (29), yeast hexokinase does not contain the consensus sequence GXGKTXG(V), either in the region comprised by HPP-50 or in other regions of the enzyme.

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