The Subunit Structure of Rabbit Skeletal-Muscle Phosphofructokinase and the Amino Acid Sequence of the Tryptic Peptide Containing the Highly Reactive Thiol Group

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1. The single highly reactive (class I) thiol group per 80000-mol.wt. subunit of skeletal-muscle phosphofructokinase was specifically carboxymethylated with iodo[2-¹⁴C]acetate, and after denaturation the remaining thiol groups were carboxymethylated with bromo[2-³H]acetate. After tryptic digestion and peptide 'mapping' it was found that the ¹⁴C radioactivity was in a spot that did not contain significant amounts of ³H radioactivity, so it is concluded that there is not a second, 'buried' cysteine residue within a sequence identical with that of the class-I cysteine peptide. 2. The total number of tryptic peptides as well as the number of those containing cysteine, histidine or tryptophan were inconsistent with the smallest polypeptide chain of phosphofructokinase (mol.wt. about 80000) being composed of two identical amino acid sequences. 3. The amino acid sequence of the tryptic peptide containing the class-I thiol group was shown to be Cys-Lys-Asp-Phe-Arg. This sequence is compared with part of the sequence containing the highly reactive thiol group of phosphorylase.

The structure of the rabbit skeletal-muscle phosphofructokinase molecule (ATP-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) has been the subject of considerable disagreement. Recent estimates for the subunit mol.wt. range between 67000 and 93000 (Tauri *et al.*, 1972; Leonard & Walker, 1972; Bloxham & Lardy, 1973; Coffee *et al.*, 1973) and the smallest active form of the enzyme comprises four of these subunits. Paetkau *et al.* (1968) presented evidence that a subunit of mol.wt. 93000 comprised four polypeptide chains of mol.wt. 23000 arranged in the form of A₃B or A₂B₂. However, Coffee *et al.* (1973) gave values for the subunit mol.wt. of 75000–85000, and by enumeration of cysteine-containing tryptic peptides suggested that each of the subunits comprised two similar, if not identical, polypeptide chains joined in such a way as to be resistant to separation under the usual denaturation conditions.

The object of the present study was first to determine whether there is any internal homology in the 80000-mol.wt. subunit. In particular, as there is a single, highly reactive thiol group per subunit (designated the class-I thiol group by Kemp & Forest, 1968), we proposed to determine whether a second thiol group in an identical amino acid sequence was 'buried' in the native structure of the enzyme. Secondly, it was proposed to determine the amino

acid sequence of the tryptic peptide containing the class-I thiol group.

Materials and Methods

Enzyme

Rabbit skeletal-muscle phosphofructokinase was prepared by a modification (Rodriguez, 1973) of the method of Kemp & Forest (1968). The specific enzyme activity, determined by the titrimetric method of Dyson & Noltman (1965), was in the range of 160–180 µmol/min per mg of protein. The concentrations of enzyme solutions were determined spectrophotometrically at 279 nm by using the value of $A_{1\text{cm}}^{1\%} = 10.2$ given by Parmeggiani *et al.* (1966). The protein gave a single band on SDS*/polyacrylamide-gel electrophoresis, in a position corresponding to a mol.wt. of 80000 ± 5000.

Materials

Trypsin treated with 1-chloro-4-phenyl-3-tosylamidobutan-2-one (Tos-Phe-CH₂Cl; batch no. 348879) was obtained from Worthington Biochemicals, Freehold, NJ, U.S.A.

Iodo[2-¹⁴C]acetate and bromo[2-³H]acetate were

* Abbreviation: SDS, sodium dodecyl sulphate.

obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Iodoacetic acid was recrystallized twice from *n*-heptane. Pyridine was redistilled from a mixture with ninhydrin. Dithiothreitol (A grade) was obtained from Calbiochem, San Diego, CA, U.S.A. Guanidine hydrochloride (grade 1) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 5,5'-Dithiobis-(2-nitrobenzoic acid) (Nbs₂, Ellman's reagent) was recrystallized from an ethanol/water mixture.

SDS/polyacrylamide-gel electrophoresis

This was carried out by the method of Weber & Osborn (1969).

Scintillation counting

This was carried out with an Intertechnique SL 40 instrument which was programmed for automatic quench correction of the dual-labelled samples. The radioactivities of the samples were determined to an accuracy of 2%.

Tryptic digestion

The carboxymethylated enzyme (prepared by the procedure described below) was suspended (2–5 mg/ml) in 1% NH₄HCO₃, adjusted to pH 8.3 with aq. NH₃, and Tos-Phe-CH₂Cl-treated trypsin, freshly dissolved in the same buffer, was added to the suspension to give a final trypsin/enzyme ratio of 1:100 (w/w). The mixture was gently shaken for 3 h at 37°C and the reaction terminated by the addition of a few drops of concentrated formic acid. The resulting clear solution was freeze-dried.

Separation of the tryptic peptides

A sample (6–7 mg) of the tryptic digest was dissolved in 100 μl of a pH 6.4 buffer (pyridine/acetic acid/water, 25:1:225, by vol.) and applied, in a thin strip, to a sheet of Whatman 3MM chromatography paper (Whatman Ltd., Maidstone, Kent, U.K.). After downward chromatography with a freshly prepared butanol/acetic acid/pyridine/water mixture (5:1:5:4, by vol.) for 24 h, the resulting chromatogram was dried at 60°C for 30 min and then subjected to electrophoresis at pH 6.4 in the second dimension. The electrophoresis was performed in water-cooled Michl tanks (see Huehns, 1968), under Varsol (white spirit, from BDH, Poole, Dorset, U.K.), for 2 h at 2 kV by the method of Huehns (1968) (for further details see Beale, 1969). The strip containing the neutral peptides (i.e. no net change at pH 6.4) was removed and sewn on to a fresh sheet of 3MM paper, which was then carefully moistened with a pH 3.6 pyridine/acetic acid/water (1:10:90, by vol.) buffer. The resultant chromatogram then underwent electrophoresis for 2½ h at 2 kV in the same buffer. After electrophoresis the chromatograms were dried at 60°C for 30 min.

Location of peptides

The chromatograms were dipped in a solution of 1% ninhydrin in acetone containing a few drops of the pH 6.4 buffer. They were dried at 60°C for 5–10 min and left to hang over conc. H₂SO₄ overnight. Peptides containing tryptophan and histidine were detected with Ehrlich's and Pauly's reagents respectively, by the methods described by Smith (1960, pp. 253, 370). Detection of the [¹⁴C]carboxymethylated peptides by radioautography was carried out by the method of Huggins (1968).

Amino acid analysis

The amino acids were analysed by using a Technicon AA1-NC1 amino acid analyser (Technicon Instruments Corp., Basingstoke, Hants., U.K.).

Isolation of the class-I thiol-containing peptide

A typical procedure was as follows: 0.5 ml of 5 mM-iodo[2-¹⁴C]acetate (10 μCi/μmol) was added to a solution containing 120 mg of enzyme in 9.5 ml of a buffer comprising 50 mM-triethanolamine hydrochloride, 1 mM-EDTA, 2 mM-fructose 6-phosphate, pH 7.1, at 25°C. After 2.5 h (4–5 half-lives) reaction in an atmosphere of N₂, the mixture was diluted by the addition of 40 ml of the ice-cold triethanolamine hydrochloride buffer and the protein precipitated by addition of solid (NH₄)₂SO₄ to 0.7 saturation. After standing for 30 min, the precipitate was collected by centrifugation at 20000g for 30 min at 4°C, resuspended in 40 ml of 1% (w/v) NH₄HCO₃, and the precipitation procedure repeated. The precipitated protein was redissolved in 8.5 ml of buffer containing 6 mM-guanidine hydrochloride, 1 mM-EDTA, 1 mM-dithiothreitol, 10 mM-NH₄HCO₃, pH 8.3, to which was added 1 ml of 0.1 M-potassium bromo[2-³H]-acetate solution (50 Ci/mol). (The extent of thiol-group reaction was monitored by addition of 5 μl portions of the reaction mixture to 1 ml of 1 mM-Nbs₂. The resultant suspensions were centrifuged for 2 min in an Eppendorf 3200 centrifuge and the absorbance of the supernatant was measured at 412 nm.) After 5.5 h the reaction was 98% complete, at which stage dithiothreitol was added to a final concentration of 2.5 mM. The resulting solution was dialysed continuously at 4°C for 2 days against 20 litres of 1% NH₄HCO₃, pH 8.3.

The protein which precipitated during dialysis was suspended in 30 ml of 1% NH₄HCO₃, pH 8.3, transferred to a 500 ml round-bottomed flask and 1.2 mg of Tos-Phe-CH₂Cl-treated trypsin added. After gentle shaking for 3 h the digestion was stopped by the addition of a few drops of formic acid and the solution freeze-dried. To ensure complete removal of the NH₄HCO₃, the peptides were redissolved in water and again freeze-dried.

Separation of the peptides

A sample (51 mg) of the digested protein was dissolved in 1% NH_4HCO_3 , pH 8.3, and applied to a column (100 cm $\times$ 1 cm) of Sephadex G-25 (fine grade), equilibrated with the same buffer.

The transmittance at 284 nm of the effluent (flow rate 20 ml/h) was monitored by using an LKB Uvicord II instrument linked to an LKB 6520-2 recorder, and 2 ml fractions were collected with an LKB 700 fraction collector. The A_{230} of the fractions was measured with a Pye-Unicam SP.800 spectrophotometer, and 20 μl samples were taken for scintillation counting. Fractions A, B, C and D (see Fig. 1) were freeze-dried twice, redissolved in about 5 ml of water and centrifuged at 5000g for 10 min to remove any insoluble material (this did not result in any loss of radioactivity). Finally the fractions were freeze-dried and stored at -20°C . Fraction B, which contained most of the ^{14}C radioactivity, was dissolved in 300 μl of the pH 6.4 buffer and 100 μl of the resulting solution was applied to a sheet of Whatman 3MM chromatography paper. The sample was electrophoresed by the techniques described above at pH 6.4 for 90 min at 2 kV, followed by chromatography for 20 h. The resultant chromatograph was radioautographed (by using a 48 h exposure). The [^{14}C]carboxymethylated peptides were located exclusively in the neutral region, which was removed for electrophoresis at pH 3.6 for 2.5 h at 2 kV and then radioautographed as before.

Results

Isolation of the tryptic peptide containing the class-I thiol group

The object of this part of the investigation was twofold. First, it was intended to isolate and deter-

mine the amino acid sequence of the tryptic peptide containing the class-I thiol group. Secondly, we wished to ascertain whether there was a second, buried cysteine residue within the subunit, in an amino acid sequence identical with that containing the class-I thiol group. It was proposed to make the class-I thiol group react with iodo[^{14}C]acetate to give a carboxymethylated derivative, and then, after unfolding, to label the remaining thiol groups with bromo[^3H]acetate. In this case, it was essential to determine the rate of reaction of the class-I thiol group with iodoacetate. This reaction was followed by measuring the decrease with time in the number of thiol groups which reacted rapidly with Nbs_2 . Fig. 2 shows photographs of time-courses of the reaction of the enzyme with Nbs_2 after 0 and 30 min reaction with 0.2 mM-iodoacetate, from which it was determined that the half-life for the carboxymethylation reaction was about 30 min. Given that the second-order rate constant for the reaction of the class-I thiol group with iodoacetate is $2.3 \text{ M}^{-1} \cdot \text{s}^{-1}$, it can be calculated that, after 2.5 h reaction under the conditions of Fig. 2, about 96% of this group should be substituted, i.e. only 4% of the class-I thiol group would then be available for labelling with ^3H radioactivity.

The procedure for the isolation of the class-I thiol-containing tryptic peptide is outlined in Scheme 1, and given in detail in the Materials and Methods section. Scheme 1 also gives the amounts of radioactivity in the various fractions.

The stoichiometry of incorporation of the carboxymethyl group in the first reaction, calculated from the amount of ^{14}C radioactivity incorporated, corresponds to 0.69 residue per subunit, which is likely to be a low estimate (see the Discussion section). After tryptic digestion of the double-labelled protein, the peptides were separated by chromatography on

Fig. 1. Gel filtration of the tryptic digest of [^{14}C]/[^3H]-carboxymethylated phosphofructokinase

The tryptic digest of phosphofructokinase, carboxymethylated first with iodo[^{14}C]acetate and then, after denaturation, with bromo[^3H]acetate (51 mg in 1 ml of 1% NH_4HCO_3 ; see Scheme 1), was applied to a column (100 cm $\times$ 1 cm) of Sephadex G-25 (fine grade) previously equilibrated with 1% NH_4HCO_3 . The elution profiles are given of transmittance at 284 nm (—), absorbance at 230 nm (----), ^3H radioactivity (····) and ^{14}C radioactivity (— · — · —). Fractions (2 ml) were collected and pooled to give solutions A, B, C and D as indicated at the top of the diagram,

Scheme 1. Outline of the procedure for double-labelling of phosphofructokinase and isolation of the class-I thiol peptide. The procedures outlined here are described in detail in the text. The class-I thiol group is designated ' S_IH ' and the remaining thiol residues as ' $(-SH)_n$ '. In the first step the high reactivity of the class-I thiol was exploited to substitute it specifically with a [^{14}C]carboxymethyl group. After denaturation, the remaining groups were carboxymethylated with bromo[^3H]acetate and the doubly labelled product was subjected to tryptic digestion. The digest was then separated into fractions A, B, C and D by chromatography on Sephadex G-25 (see Fig. 1). Electrophoresis at pH 6.4 and chromatography followed by electrophoresis at pH 3.6 gave ^{14}C -containing peptides. The radioactivities of the various fractions are given and the significances of these values are given in the text.

Fig. 2. Reaction of the class-I thiol group of phosphofructokinase with iodoacetate

The reaction was followed by determination of the concentration of fast-reacting thiol groups at different times by reaction with Nbs_2 . A solution of enzyme (1 mg/ml in pH 7.1 buffer; see below) was placed in syringe 1 of the stopped-flow apparatus and sodium iodoacetate added to a final concentration of 0.2 mM. The second syringe contained 1.0 mM- Nbs_2 reagent in the pH 7.1 buffer. Time-courses of the reaction of the enzyme thiol groups with Nbs_2 were recorded at different times [time-courses at (a) 0 and (b) 30 min are shown] and the concentration of unchanged class-I thiol groups was determined from the amplitude of the fast phase of the reaction. The pH 7.1 buffer comprises 0.1 M-triethanolamine hydrochloride, 1 mM-EDTA and 2 mM-fructose 6-phosphate, pH 7.1 at 25°C. The parallel lines in the upper part of the traces are the continuations of the time-courses, obtained by allowing the time-base of the oscilloscope to run continuously after the initial triggering.

a column (100 cm $\times$ 1 cm) of Sephadex G-25. The elution profiles of absorbance at 230 nm, transmittance at 284 nm and the ^{14}C and ^3H radioactivities from the resulting chromatogram are shown in Fig. 1, which shows that most of the ^{14}C radioactivity is in a single peak, well separated from most of the ^3H radioactivity.

The eluate from the column was divided into four fractions, designated A, B, C and D (see Fig. 1), of which fraction B contained most of the ^{14}C radioactivity, i.e. the peptide containing the class-I thiol group is in this fraction. The carboxymethylated peptides in fraction B were then separated by electrophoresis at pH 6.4 and paper chromatography (see the Materials and Methods section). Radioautography of the resulting chromatogram showed that the ^{14}C radioactivity was confined to the 'neutral' region, so the neutral peptides were then resolved further by electrophoresis at pH 3.6. Radioautography indicated the presence of two ^{14}C -labelled peptides designated X and Y (see Fig. 3). The amino acid composition of peptides X and Y, together with their ^3H and ^{14}C radioactivities, were determined (see Scheme 1). Both peptides contained about one residue each of aspartic acid, arginine, lysine and phenylalanine, and 0.4–0.6 residue of *S*-carboxymethylcysteine.

We conclude that the presence of two peptides is most probably accounted for by partial oxidation of a single peptide (Harris, 1967), and this is supported by the observation that the $^{14}\text{C}/^3\text{H}$ ratios of the two peptides are closely similar.

By the following argument it can be demonstrated that there is not a second, 'buried' class-I thiol-

containing peptide sequence in the phosphofructokinase subunit. (i) In the experiment described in Scheme 1, the incorporation of one ^{14}C -labelled residue per μmol of subunit would give 3.4×10^5 d.p.s. (calculated from the specific radioactivity of the iodo[^{14}C]acetate). (ii) The incorporation of one ^3H -labelled residue per μmol of subunit would give 1.5×10^6 d.p.s. (from the specific radioactivity of the iodo[^3H]acetate). (iii) Hence, if there were two peptides with an equivalent sequence, one containing *S*-[^{14}C]carboxymethylcysteine and the other *S*-[^3H]carboxymethylcysteine, then the ratio of $^3\text{H}/^{14}\text{C}$ radioactivity would be 4.4. If there was not a buried thiol group, the kinetics of the reaction would predict a ratio of 0.18 (calculated from the time of reaction and the second-order rate constant; see above). (iv) The observed ratios were 0.60 for peptide X and 0.56 for peptide Y. We therefore conclude that the sequence containing the class-I thiol group occurs only once within the polypeptide chain of mol.wt. 80000. The difference from the predicted value of 0.18 can be ascribed to the reaction of the non-class-I thiol groups with iodo[^{14}C]acetate.

Amino acid sequence of the class-I thiol-containing peptide (X)

The following sequence was determined by the methods described by Hartley (1970): Cm-Cys-Lys-Asp-Phe-Arg. The failure of trypsin to catalyse the cleavage of the Lys-Asp peptide bond can be attributed to the two adjacent negatively charged residues (e.g. see Hirs *et al.*, 1956).

Tryptic 'mapping' of phosphofructokinase

Tryptic hydrolysates of the [^{14}C]carboxymethylated enzyme were prepared as described in the Materials and Methods section. A sample (about 6mg) of the freeze-dried digest was dissolved in 150 μl of the pyridine/acetic acid/water buffer, pH 6.4, and the resultant solution subjected to downward paper chromatography developed by elution with butanol/acetic acid/pyridine/water (5:1:4:5, by vol.) for 24h.

After concentration of the peptides into a narrow band by careful wetting of the chromatogram, electrophoresis was carried out at pH 6.4 in the second dimension for 2h at 2kV. The region of the chromatogram containing the neutral peptides (i.e. no net charge at pH 6.4) was cut out and sewn on to a second sheet of Whatman 3MM paper, and further electrophoresis carried out in buffer, pH 3.6, for 2.5h at 2kV. Ninhydrin-reacting, tryptophan-containing and histidine-containing peptides were then located on the resultant chromatograms and ^{14}C -containing peptides located by radioautography (see the Materials and Methods section).

A summary of the results is shown in Fig. 3, which shows that there were about 86 ninhydrin-positive spots, comprising about 39, 27 and 20 spots in the basic, neutral and acidic regions respectively. Of these peptides, 13 contained histidine (one of which contained more than one residue), and six peptides contained tryptophan (two of which appeared to contain more than one residue).

The composition of the *S*-carboxymethylcysteine-containing peptides indicated that there were 10–12 unique thiol-containing sequences. However, most of these peptides gave two ninhydrin-reactive spots

resulting from partial oxidation (see above and the Discussion section). Phosphofructokinase contains 42 lysine and 49 arginine residues per 80000 daltons (Simpson, 1975). Therefore, given that the mol.wt. of the phosphofructokinase subunit is 80000, a tryptic digest should contain 92 peptides. The present results indicate the presence of at least 74 peptides (allowance being made for duplication of spots owing to partial oxidation of *S*-carboxymethylcysteine residues). This number of peptides, which is significantly greater than that reported by other workers (Paetkau *et al.*, 1968; Tauri *et al.*, 1972), indicates that it is unlikely that the 80000-mol.wt. subunit comprises two chains of identical sequence. This conclusion is supported further by the number of tryptophan residues accounted for (i.e. theoretical: ten residues per 80000 daltons; observed: six peptides, two containing more than one residue) and the number of histidine residues detected (i.e. theoretical: 16 residues per 80000 daltons; observed: 13 peptides, one containing more than one residue). This argument is subject to the reservations raised in the Discussion section.

Discussion

The results of this study indicate that the smallest subunit of the phosphofructokinase molecule comprises a single polypeptide chain of mol.wt. about 80000 ± 5000 . There is no evidence for any internal repeats of sequence within this chain, and the single highly reactive class-I thiol group per 80000 daltons is unique, in that there is not a second, 'buried', cysteine thiol group in the polypeptide chain within an identical sequence. Indeed, from amino acid analysis, all the cysteine-containing peptides were

Fig. 3. Peptide 'maps' of a tryptic digest of [^{14}C]carboxymethylated phosphofructokinase

The sample of trypsin-digested *S*-[^{14}C]carboxymethylated enzyme used for the peptide 'mapping' was prepared as described in the Materials and Methods section. Samples (6–7 mg) of the digest in 100–150 μl of buffer were applied to the paper for electrophoresis at pH 6.4. After electrophoresis (in the vertical dimension on the diagrams), paper chromatography was carried out in the second (horizontal) dimension and the acidic and basic peptides are shown in (a) and (b). The neutral peptides were further resolved by electrophoresis at pH 3.6 to give 'map' (c) (for details see the Materials and Methods section). The solid outlines in the drawings represent ninhydrin-staining spots, and other spots containing histidine (H), tryptophan (T) and ^{14}C radioactivity (C) are also shown. A representation such as T $\times$ 2 indicates that a peptide stains sufficiently strongly to suggest that it may contain more than one tryptophan residue.

unique and could not give a sequence homologous with that of the class-I thiol-containing peptide (Simpson, 1975). The amino acid composition of the tryptic peptide F, isolated by Coffee *et al.* (1973), indicates that it corresponds to peptide X of our study (i.e. the peptide containing the class-I thiol group).

The evidence on which the above conclusions are based is as follows. First, the number of tryptic peptides observed (about 74), although less than predicted from the amino acid analysis and a mol.wt. of 80000 (about 92), is considerably larger than both that predicted for a duplicated sequence (i.e. 47) and the numbers observed by Paetkau *et al.* (1968) and Tauri *et al.* (1972) (i.e. 43–48 and 50 respectively). Direct comparison of tryptic 'maps' is not possible, as each group of workers used different procedures. However, it is probably significant that larger amounts of peptide were applied to each chromatogram in the present study, and this enabled detection of peptides that migrate towards the anode (acidic peptides), which stain poorly with ninhydrin and were not detected at lower protein concentrations. It is noteworthy that most of the *S*-carboxymethylated peptides were located in this region. We found that these peptides were relatively large (e.g., see Fig. 1) and prone to precipitation at low pH values.

Secondly, the numbers of *S*-carboxymethyl-, histidine- and tryptophan-containing peptides were also greater than reported previously [e.g. by Paetkau *et al.* (1968) and Coffee *et al.* (1973)] and again would be inconsistent with a polypeptide chain containing a duplication of sequence.

As indicated by Harris & Hindley (1965), estimation of the size of a polypeptide chain by tryptic 'mapping' in conjunction with amino acid analysis is subject to a number of sources of error. In particular the oxidation of *S*-carboxymethylcysteine and methionine residues can lead to a duplication of peptides (a correction has been made for this with the *S*-carboxymethylcysteine peptides). Another potential problem is that of incomplete tryptic digestion, which is evident in the present study in that peptide X contains both an arginine and a lysine residue. However, we consider the total number of tryptic peptides, as well as the numbers of tryptic peptides containing cysteine, histidine and tryptophan, to be both so much greater than expected for a duplicated sequence that they could not have arisen from these sources or error.

The conclusions presented above agree with the findings of Walker *et al.* (1976) that 15 of the 16 cysteine residues of the subunit of phosphofructokinase of mol.wt. 85000 occur in unique sequences, and that the total number of arginine-containing tryptic peptides (55) is close to that expected for a subunit of mol.wt. 85000 (i.e. 53) on the basis of the amino acid composition. Walker *et al.* (1976) con-

cluded that the amino acid sequence of the subunit of mol.wt. 85000 appears to be unique along its entire length.

The amino acid sequence of the peptide containing the reactive (class I) thiol group reported here corresponds to the reactive cysteine-containing tryptic peptide designated TD4 by Walker *et al.* (1976). Our studies demonstrate that this sequence occurs only once in the subunit of mol.wt. about 80000.

We have compared the sequence of the class-I thiol peptide with the sequences of peptides containing highly reactive thiol groups in other enzymes. The only sequence bearing any similarity was that from phosphorylase (Gold & Blakeman, 1970), i.e. ...Gly-Cys-Arg-Asp-Pro...

(cf. $\begin{matrix} \text{Lys} \\ \text{Arg} \end{matrix}$ -Cys-Lys-Asp-Phe-...)

from phosphofructokinase). It is noteworthy that the reactive thiol groups in both enzymes are close to the regulatory nucleotide-binding sites (Jones *et al.*, 1972, 1975; Gold & Blakeman, 1970) and that both thiol groups undergo changes in reactivity on the binding of regulatory nucleotides (Kemp, 1969; Sanner & Tron, 1975). However, although it is tempting to ascribe a role to these groups in the allosteric transitions in these enzymes it should be noted that Jones *et al.* (1972) reported that phosphofructokinase underwent only small changes in kinetic properties on spin-labelling the enzyme at the class-I thiol group, and later reported that the spin-labelled enzyme still appears to bind ATP at both the catalytic and regulatory sites (Jones *et al.*, 1975).

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