observations of the helix-coil transition of poly G, which were carried out with the product obtained from such polymerizations. They also provide an explanation for the priming effect toward poly G synthesis of those oligonucleotides that serve as chain initiators, and for the lack of such priming by those oligonucleotides that are not incorporated in the product (cf. (4)). When oligonucleotides are incorporated, the presence of a terminal sequence of non-guanine residues will markedly weaken the interaction of poly G strands and therefore allow longer chains to be synthesized before the enzyme is inhibited. It follows, then, that oligonucleotides not incorporated will not be similarly effective. In any case, it does not appear that there is an absolute requirement in poly G synthesis for oligonucleotides that are incorporated.

Observations on the physical properties of poly G and its interaction with poly C are being reported elsewhere.

REFERENCES


Identification of Phosphohistidine in Digests from a Probable Intermediate of Oxidative Phosphorylation*

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Recent studies on mitochondrial components rapidly labeled after addition of Pi32 allowed detection of an unidentified, protein-bound phosphate that appeared to be an intermediate in oxidative phosphorylation (1). The principal purpose of the present communication is to report results of fractionation and characterization studies which have led to the identification of phosphohistidine1 in digests of the unidentified phosphate.

Freshly isolated mitochondria were used for fractionation studies because attempts to use whole tissue as starting material were not successful. Mitochondria prepared from ground bovine liver initially subjected to a brief Waring Blendor treatment in 0.25 M sucrose, 1 mM EDTA, pH 7.4, gave yields of the unidentified phosphate from Pi32 comparable to those from rat liver mitochondria prepared by conventional procedures. Interfering impurities in commercial Pi32 were removed by absorption of the Pi32 on an anion exchange resin, Amberlite CG-4B, followed by elution with 0.05 M NH4OH. Mitochondria from about 100 g of liver were incubated for 30 seconds with Pi32 under conditions as described previously (1). Then, 2 volumes of a cold solution containing 7.5 M urea and 0.3 M NH4OH were added to give apparent solubilization of the mitochondria. Batches from several incubations were pooled and passed through anion exchange columns (Dowex 1-Cl-, 8 to 10% cross-linked, 200 to 400 mesh), and the effluent was subjected to acetone fractionation at approximately 4°C. The moist acetone precipitate containing most of the radioactivity was treated with 2 N NaOH at 70°C to solubilize completely the Pi32-containing component.

Solubilized material from the alkaline digestion was passed through columns of Sephadex G-25 previously equilibrated with 0.01 N NaOH. Radioactive fractions collected after the bulk of the protein, but prior to most of the NaOH, salts, and Pi32, were pooled and diluted, and the radioactive component was adsorbed on Dowex 1-Cl-. The column was eluted with a linear gradient of 0 to 1.0 M NaSO4, pH 9.0, which separated the radioactive component from Pi32. The radioactive component was then eluted by a 0 to 0.5 M NaSO4 gradient at pH 9.0. Removal of sulfate was accomplished by careful evaporation, followed by separation of solid Na2SO4 or by precipitation of the sulfate with Ba(OH)2. Digestion of such concentrates with a crude mold protease (Pronase) at pH 8.0, followed by separation on Sephadex G-25, gave a product which, when chromatographed on Dowex 1 OH-, showed a single radioactive peak in addition to a small Pi32 peak.

Properties of fractions obtained in this manner suggested that the phosphorylated substance was distinct from any material previously reported to be present in living systems. Phosphohistidine and phosphorylated imidazoles have been prepared previously (3-9), and properties of the unidentified component appeared consistent with its possessing a phosphorylated imidazole structure. Rates of hydrolysis of purified fractions were measured by the extraction procedure of Berenblum and Chain.

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1 Phosphorylated imidazole derivatives may be designated as phosphonates, e.g.

\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{P} & \quad \text{OH} \\
\text{OH} &
\end{align*}
\]

as imidazolyl 1-phosphonate (2). Biochemical relationships of histidine with a phosphoryl on a ring nitrogen are more appropriately indicated by the designations 1- or 3-phosphoryl histidine, and phosphohistidine is used as a trivial name for such monophosphoryl derivatives.
FIG. 1. Chromatographic separation of a purified radioactive component from phosphohistidine. A fraction prepared as indicated in the text with Pronase digestion was chromatographed on a Dowex 1-(OH) column (1 × 9 cm), with 0 to 1.5 M NaHCO₃- CO₂, pH 8.5, for elution. Presence of Pᵢ² and the unidentified phosphate was measured by radioactivity, and phosphohistidine was measured by determination of acid-hydrolyzable phosphate. X—X, counts per minute; O—O, micromoles of hydrolyzable phosphate.

FIG. 2. Apparent chromatographic identity of a purified radioactive component with synthetic phosphohistidine following strong alkali digestion. Conditions were the same as in Fig. 1, except that the unidentified phosphate was hydrolyzed with 3 N NaOH as described in the text. X—X, counts per minute; O—O, micromoles of hydrolyzable phosphate.

(10), performed rapidly, to determine quantitatively release of Pᵢ² from the unidentified phosphate. Rate of release of Pᵢ from added synthetic phosphohistidine (3, 11) was found to be identical with the release of Pᵢ² from the unidentified phosphate at pH 7, pH 3.8, and in 2 N acid, and further, hydrolysis of both the unidentified phosphate and phosphohistidine was similarly accelerated by molybdate or pyridine.

Cochromatography of a fraction purified as outlined above with phosphohistidine showed differences in their migration properties, as shown in Fig. 1. The possibility existed that amino acids or other residues still attached to the radioactive product caused it to migrate more slowly than phosphohistidine. Thus, a similar fraction was subjected to a comparatively rigorous alkaline hydrolysis (3 N NaOH, 100°, 2 to 3 hours in a sealed tube), during which approximately 30% of the radioactivity was liberated as Pᵢ². The remaining organic phosphate still showed hydrolytic properties for Pᵢ² release like those of synthetic phosphohistidine. After this treatment, however, migration of the unidentified phosphate became identical with that of synthetic phosphohistidine, as shown in Fig. 2. The slight deviation on the tailing side of the peak probably represents impurities in the synthetic phosphohistidine. Identical
The striking similarity in three-dimensional conformation of the individual chains in hemoglobin and myoglobin (1, 2), together with their similar and perhaps unique titration behavior (3, 4), suggests a causal relationship between their secondary structures. The conditions governing the presence of the phosphorylated imidazole structure are complex and require further study. Additional evidence for the participation of the phosphohistidine moiety in oxidative phosphorylation has been obtained with ATP. These experiments have shown production of the unidentified phosphate from ATP independent of passage through P1; previous experiments (1) have shown production from P1 independent of passage through ATP. Concentrates containing solubilized radioactive fractions when added to mitochondria are converted to P1 and ATP, but the nature of this conversion is not yet established. Known enzyme and non-enzymatic reactions (5-9, 15, 16) of phosphorylated imidazoles are consistent with such structures as intermediates in enzyme phosphorylation reactions.

In summary, a previously unidentified and rapidly labeled phosphate fraction of mitochondria has been degraded to yield phosphohistidine. This is the first observation of the presence of a phosphorylated imidazole structure in material isolated from living systems. The conditions governing the presence of the fraction suggest that it represents an intermediate in oxidative phosphorylation.

REFERENCES


Role of Heme in the Titration of Sperm Whale Myoglobin*

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The striking similarity in three-dimensional conformation of the individual chains in hemoglobin and myoglobin (1, 2), together with their similar and perhaps unique titration behavior (3, 4), suggests a causal relationship between their secondary structures.
CORRECTION

In the Preliminary Communication by P. D. Boyer, M. DeLuca, K. E. Ebner, D. E. Hultquist, and J. B. Peter on page PC3308, Vol. 237, No. 10, October 1962, several lines of type were omitted immediately preceding the structural formula in the first column. The entire column is reprinted in the adjoining column so that readers may cut out the corrected column and paste it in the appropriate place on page PC3308 of the October issue.

chromatographic behavior of the radioactive phosphate and phosphohistidine was also observed with Na$_2$SO$_4$ or NaHCO$_3$. Na$_2$CO$_3$ elution at approximately pH 9. The product liberated by strong alkali digestion also migrated with phosphohistidine on paper chromatography with propanol-ethanol-water-ammonia (12), and on paper electrophoresis with 2% sodium borate buffer.

Paper chromatographic separation of material obtained essentially as described before with strong alkali, but without Pronase digestion, gave sufficient product for direct detection of the phosphate compound on paper by reaction with ninhydrin or the Pauly diazo reagent (13) used for histidine detection. Like synthetic phosphohistidine, the isolated fraction gave a Pauly test reaction only after acid hydrolysis, indicating attachment of the phosphate to the imidazole ring. Acid hydrolysis before paper chromatography resulted in the appearance of a Pauly-reactive material that comigrated with authentic histidine, and the disappearance of the material which migrated with phosphohistidine.

Fractions were further purified by elution from wide paper chromatograms to yield sufficient material for direct measurement of phosphate and histidine content by micro procedures. Mild acid hydrolysis of one fraction obtained in this manner gave 2.7 mmoles of phosphate and 2.3 mmoles of histidine; a second fraction gave 5.4 mmoles of phosphate and 4.5 mmoles of histidine. The yields of phosphate and histidine are close to the 1:1 ratio expected for phosphohistidine considering the limitations of methods and purity of the samples. These results together with the other findings give convincing evidence for the identity of the isolated product with phosphohistidine. Absolute identification as phosphohistidine, however, in contrast to some unrecognized but closely similar substance, must await isolation of larger amounts of the products. Whether the natural as well as the synthetic product consists solely or principally of the 3-phosphoryl derivative of histidine, as depicted below, or the L-isomer is not known. Preliminary experiments indicate a probable presence of isomers in the synthetic product.

\[
\begin{align*}
\text{CH}_3-\text{CH-COO}^- \\
\text{O}^-\text{P} \text{O}^- \\
\text{NH}_3^+ \\
\text{3-Phosphoryl histidine}
\end{align*}
\]

That the phosphorylated imidazole structure probably exists in the mitochondria and does not result from a transphosphorylation after incubation is indicated by the findings of a similar material whether the alkaline urea, cold ethanol, neutral urea-Triton X-100 (1), or 3:1 ethanol-diethyl ether is used to stop mitochondrial enzyme reactions. Further, the unidentified phosphate as initially detectable in a protein-bound form has hydrolytic properties very similar to those of phosphohistidine. Present evidence is consistent with the phosphorylated imidazole being present on a histidine moiety of a polypeptide or protein. The specific activity of the phosphate isolated from purified concentrates is approximately the same as that estimated for the intramitochondrial P$_i$. This suggests that the rapidly labeled phosphohistidine moiety described herein may be the only protein-bound phosphohistidine present in mitochondria.

Additional evidence for the participation of the phosphohistidine moiety in oxidative phosphorylation has been obtained.
Identification of Phosphohistidine in Digests from a Probable Intermediate of Oxidative Phosphorylation

P. D. Boyer, M. DeLuca, K. E. Ebner, D. E. Hultquist and J. B. Peter