An Energy Rich Phosphate from 2-Methylimidazole through Nonenzymatic Photophosphorylation*

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Sensitized photooxidation of imidazoles in aqueous solution and in the presence of inorganic phosphate afforded organic phosphates with yields up to 30%. The major phosphorylated product of oxidation of 2-methylimidazole was isolated and identified as the mono-phosphate of 4,5-dihydroxy-2-methylimidazoline with pKa values of 2.0, 5.2, and 9.8. The compound is energy rich in aqueous solution and hydrolyses in alkaline or neutral solutions irreversibly (\( \Delta H_{\text{hyd}} = -9.8 \text{ kcal/mole} \)). For the hydrolysis at pH 7 an activation-enthalphy of 25 kcal/mol and -entropy of 8 cal/deg.mole was observed. Be\(^{2+}\) ions catalyse the hydrolysis.

Phosphorylation coupled to photoredox reactions is a fundamental process in the acquisition of solar energy by the biosphere. In our search for non-enzymatic models under the aspect of the origin of biological and prebiotic systems [1], we reported previously that inorganic phosphate can be trapped in an energy rich form during the oxidation of imidazoles with OH-radicals [2, 3]. The major phosphorylated product from the oxidation of 2-methylimidazole was isolated and identified as the O-monophosphate of 4,5-dihydroxy-2-methylimidazoline (DHMIP) [3]:

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{H} \\
\text{HO} & \quad \text{OPO}_3\text{H} \\
\text{CH}_3 & \quad \text{O}
\end{align*}
\]

This compound has now been prepared using sensitized photooxidation of 2-methylimidazole with molecular oxygen. Table I shows the yields of bound phosphate obtained from different substrates and using different sensitizers after 45 min of illumination.

Materials and Methods

The assay for bound phosphate involved isopropyl acetate extraction of P\(_1\) as molybdate complex and

\[\text{H}_2\text{P}_2\text{O}_{7}^{-}\text{OPO}_3\text{H} \quad \text{DHMIP}\]

This compound has now been prepared using sensitized photooxidation of 2-methylimidazole with molecular oxygen. Table I shows the yields of bound phosphate obtained from different substrates and using different sensitizers after 45 min of illumination.

** Professor A. Butenandt to his 80th birthday.

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1 Bioids XI, X: J. Chromatogr. 244, 281 (1982), IX: see ref. [3].
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subsequent extraction of P\(_1\) liberated by short alkalization [3].

The pH decreased from 6.5 to 6.2 during illumination. In all experiments there was some bleaching of the sensitizers (up to 75%); peroxides and H\(_2\)O\(_2\), which have been detected by iodometry, may cause the bleaching, since it was reduced by addition of small amounts of MnO\(_2\).

For the preparation of crystalline DHMIP 5.15 g 2-methylimidazole was dissolved in 75 ml 0.5 M phosphoric acid. The obtained pH of 6.9 was held constant during the reaction by addition of a 2-methylimidazole solution (5 M) using a pH-stat. 1.5 ml of a hematoporphyrin solution (4 mM) was added at the beginning and additional solution was supplemented during the reaction at a rate of 0.15 ml/h. The reaction vessel was cooled from outside to 0 °C using a cryostat and illuminated for 24 h by two projector lamps (each 100 W) while bubbling oxygen through the solution.

The reaction mixture was decolorized with active charcoal and acidified with trifluoroacetic acid in order to prevent hydrolysis. At this stage the assay for bound phosphate [3] gave yields of 20 to 30% of the initial H\(_2\)PO\(_4\). After freeze-drying the residue was dissolved in 20 ml water and precipitated by addition of cold methanol (200 ml) and cold acetone (250 ml). After 2 h at —20 °C the mother liquor was decanted from the (sometimes oily) precipitate and the precipitate was triturated and washed with several portions of cold methanol and ether.

P NMR (Bruker WH 270 FT spectrometer with Bruker Aspect 2000 computer, 109.32 MHz) revealed two organic phosphate fractions and residual P\(_1\) (Fig. 1). The residue was dissolved in 15 ml water, transferred onto an 25 X 3 cm anion exchanger column (Dowex 1 X 8) in acetate form, and eluted with water at 0 °C at a rate of 3 ml/min. The eluate was collected in tubes prefiltered with 0.1 ml trifluoroacetic acid.
Table I. Yields of bound phosphate in % of inorganic phosphate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>conc. (mol/l)</th>
<th>Sensitizer</th>
<th>conc. (10^4) (mol/l)</th>
<th>(H_2PO_4^-) conc. (mol/l)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Methylimidazole</td>
<td>0.14</td>
<td>Methylene blue</td>
<td>1.0</td>
<td>0.26</td>
<td>0.7</td>
</tr>
<tr>
<td>2-Methylimidazole</td>
<td>0.14</td>
<td>Methylene blue</td>
<td>1.0</td>
<td>0.26</td>
<td>7.2</td>
</tr>
<tr>
<td>Pyrimidine</td>
<td>0.14</td>
<td>Methylene blue</td>
<td>1.0</td>
<td>0.26</td>
<td>0.7</td>
</tr>
<tr>
<td>Histamine</td>
<td>0.14</td>
<td>Methylene blue</td>
<td>1.0</td>
<td>0.26</td>
<td>3.1</td>
</tr>
<tr>
<td>Histidin</td>
<td>0.14</td>
<td>Methylene blue</td>
<td>1.0</td>
<td>0.26</td>
<td>1.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.14</td>
<td>Hematoporphyrine</td>
<td>2.0</td>
<td>0.26</td>
<td>2.7</td>
</tr>
<tr>
<td>2-Methylimidazole</td>
<td>0.32</td>
<td>Rose bengal</td>
<td>1.0</td>
<td>0.18</td>
<td>11.4</td>
</tr>
<tr>
<td>2-Methylimidazole</td>
<td>0.32</td>
<td>Safranin T</td>
<td>3.0</td>
<td>0.18</td>
<td>2.7</td>
</tr>
<tr>
<td>2-Methylimidazole</td>
<td>0.32</td>
<td>Proflavin</td>
<td>1.0</td>
<td>0.18</td>
<td>2.1</td>
</tr>
<tr>
<td>2-Methylimidazole</td>
<td>0.25</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.4*</td>
</tr>
</tbody>
</table>

Illumination: 45 min in 5 ml vials at 12 °C using two projector lamps of 150 W each; oxygen was bubbled through the solution. Initial pH was 6.5;

* illuminated for 5 h at 0 °C using a Hg-immersion lamp.

Elemental analysis for \(C_4H_9O_5N_2P\)

Found C 24.42 H 4.78 O 40.4 N 13.83 P 15.4.
Calcd C 24.49 H 4.62 O 40.79 N 14.08 P 15.79.

\(^1\)H NMR (0.1 M \(D_2SO_4/D_2O\)): A doublet at 5.65 (\(J\ 7\) Hz), one singlet at 5.54 and one singlet at 2.4 ppm rel. TMS (integral 1:1:3) [5]. Titration of DHMIP showed three dissoziable groups with \(pK_1 = 2, pK_2 = 5.2\) for the phosphate part and \(pK_3 = 9.8\) for the imidazoline part of the molecule, in accordance with electrophoretic net charge at different pH values as well as with the changes of the shift of the \(P\) NMR signal at these pH values. DHMIP decomposes in aqueous solution producing a yellow color. Below pH 5 it can be kept for hours, but reacts almost instantaneously in alkaline solution. Preliminary calorimetric measurements of the heat of hydrolysis at 10 °C and pH 12 in phosphate buffer gave \(\Delta H_{\text{hyd}} = -9.49 \text{ kcal/mol}\) (measured during the first 15 min and corrected for the heat of solution). In the UV spectrum (Fig. 2) the maximum at

Results

Two organic phosphate fractions (maxima at 90 and at 250 ml) were obtained, \(P_1\) remained on the column. The 1st fraction could so far not be purified further because of its instability. The second fraction, from 200 to 300 ml, was lyophilized (630 mg), dissolved in a minimum amount of water and decolored with charcoal; slow addition of acetone precipitated 580 mg of crystalline DHIMP identical with preparations obtained using Fenton’s reagent [3] or bromine [4] as oxidant.

Fig. 2. UV-spectrum of DHMIP. A: freshly dissolved in \(H_2O\); B: after 4 h at room temperature.
215 nm approaches a time independent value, whereas a persisting slow shift at 250 nm probably reflects secondary reactions. The change on both wavelengths follows simple pseudo first order kinetics.

Above pK$_3$ (9.8) hydrolysis is catalyzed by OH$^-$. Between pK$_3$ and pK$_2$ the rate constants are more or less pH independent. Here we found 25.1 kcal/mol for the enthalpy and 8 cal/mol degree for the entropy of activation. Mg$^{++}$, Mn$^{++}$ and Cu$^{++}$ have little effect, but Be$^{++}$ ions strongly catalyze the hydrolysis in the neutral region.

Discussion

The mechanism of DHMIP formation by photophosphorylation is not clear. Oxygen is required for phosphate binding. Attempts for an anaerobic phosphorylation of 2-methylimidazole using different sensitizers and methylviologen as electron acceptor did not succeed. Such a reaction should be of interest as a model of prebiotic photophosphorylation [1]. Also the superoxide ion appears not to be involved since superoxide dismutase had no effect onto the yield.

On the other hand addition of small amounts of singlet oxygen quenchers like NaN$_3$ or Ni$^{++}$ ions caused a marked decrease of the yield of phosphorylation. For a further verification of singlet oxygen participitation, this species was generated chemically in an additional experiment according to

$$\text{H}_2\text{O}_2 + \text{OCl}^- = \text{Cl}^- + \text{H}_2\text{O} + \text{O}_2.$$  

The P NMR spectrum of a solution of 2-methylimidazole and inorganic phosphate in the presence of chemically generated $^1$O$_2$ showed the formation of the same phosphorylated products. Thus the initial step is probably an attack of singlet oxygen on the imidazole ring as postulated by different authors [6-8], producing 4.5 dioxetane which is susceptible for an attack of nucleophiles, leading to a spectrum of products. As a possible mechanism for the formation of DHMIP, we suggest therefore a nucleophilic attack of inorganic phosphate to the 4.5 dioxetane intermediate.

Since 50 years after the discovery of ATP by Lohmann [9] the mechanism of the ubiquitous biological phosphorylations is still obscure, the easy and relatively high yield coupling in excited state (radical [1] or photochemical) reactions suggests alternative theoretical and experimental approaches to the old problem of the intriguing similarity of oxidative and photochemical phosphorylations.

A similar case of a biochemical oxidative phosphorylation probably involving microsomal oxidases and affording hydroxykynurenine phosphate from tryptophane and from kynurenin has been observed by Butenandt's group at Tübingen as early as 1952 [10].