Nonenzymatic Formation of Energy Rich Phosphates in the Course of Imidazole Oxidation with Fenton's Reagent*

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Oxidation of substituted imidazoles with modified Fenton's reagent (Fe(II), ascorbic acid, EDTA and H₂O₂) in aqueous solution at pH 5–7 in the presence of inorganic orthophosphate gives high yields of an unknown bound phosphate, in the case of 2-methylimidazole up to 35% based on inorganic phosphate. The dependence of the yields on reaction parameters and the hydrolytic behaviour of the compound was studied. The products are unstable in alkaline medium and fairly stable in acid solution (half-life period ca. 1 h at 25 °C in 1 N perchloric acid). Similar bound phosphate was also observed using autoxidation with O₂ instead of H₂O₂.

Oxidative phosphorylation and photophosphorylation are fundamental processes in living organisms in which energy is conserved in energy rich organic phosphates serving as standardized energy suppliers in endergonic biochemical reactions. The importance of similar processes providing the thermodynamical driving force for the selforganization of life-like systems in a prebiological scenario has been stressed by Decker [1–3]. Since thermodynamically such systems are dissipative structures, such selforganization should be expected at or near the site where on Earth we have maximal energy dissipation i.e. the dissipation of light energy [4–5]. This rationale suggested a search for an organized production of compounds with high redox- or group potentials through photoredox reactions in an open systems as a thermodynamically feasible source of selforganization. Since in sensitized photoredox reactions intermediates of high reactivity are essential, our first approach was a study of reaction products of high energy radicals in the presence of inorganic phosphate. For this purpose we chose Fenton's reagent, which is one of the most powerful noninorganic oxidizing agents, the actual oxidizing species being the hydroxyl radical formed from hydrogen peroxide and ferrous ion [6]:

\[ \text{H}_2\text{O}_2 + \text{Fe}^{\text{II}} - \text{EDTA} = \text{Fe}^{\text{III}} - \text{EDTA} + \text{OH}^- + \text{OH}^- \]

Materials and Methods

Imidazole (p.a.) was obtained from Merck, 1-methylimidazole (p.a.) and 4-hydroxymethylimidazole-hydrochloride from Riedel de Haen, 2-methylimidazole from Sigma, USA, pyrindine (98%) from Ega, histamine · 2 HCl (99%) and imidazole-4-acetic acid · Na-salt from Serva, isopropylacetate from Baker. All other reagents were of reagent degree.

Reaction conditions

In a typical experiment the complete system contained the following components (μmoles in 1 ml of total volume) at pH 4–6 and 0 °C:

- Na₂HPO₄ · 2H₂O 100
- FeSO₄ · 7H₂O 4
- ascorbic acid 40
- EDTA 20 and substrate 40

The solution was cooled with ice and the reaction was initiated by the addition of 50 μl of 30% H₂O₂. After two minutes the phosphate determination was started.

Quantitative determination of bound phosphate

200 μl of the reaction mixture was transferred into a tube containing 1.5 ml HClO₄ (10%) and 0.3 ml Na₂MoO₄ (1.2 M) in the cold (0 °C). Inorganic phosphate was extracted four times as the molybdate
complex using 4 ml portions of isopropyl acetate. Fast phase separation was enforced by centrifugation for 1 min. Under these conditions even labile phosphates remain mainly unhydrolysed in the aqueous phase [7]. The fourth extraction was usually free of inorganic phosphate. Bound phosphate in the aqueous phase was then hydrolyzed by heating to 100 °C for ten minutes. After cooling to room temperature any newly formed phosphate was obtained (to 99% [7]) by an additional extraction.

For a quantitative determination 0.1 ml of freshly prepared 0.5% SnCl₂ • 2H₂O in methanol was added to 2 ml of the organic phase and the extinction of molybdene blue was determined at 691 nm using an Eppendorf photometer. Since the bound phosphates obtained in our system were almost immediately hydrolyzed at pH values greater than 9, in later experiments we omitted the heating step for hydrolysis; instead after the fourth extraction step, we increased for 30 sec the pH of the aqueous phase to pH 12–14 using NaOH, and acidified the solution again to pH 1 with HClO₄.

This method allows the detection of 0.1% of bound phosphate in the presence of inorganic phosphate.

**Column chromatography**

A column (3 × 25 cm) with Dowex 1 × 8 (200 to 400 mesh) anion exchange resin (Serva) was transformed into the acetate form by washing with 2 M NaOH and 2 M CH₃COOH. After equilibration with 0.05 M CH₃COOH 3.4 ml of a reaction mixture of 1.5 mmoles Na₂HPO₄ • 2H₂O, 2.5 mmole 2-methylimidazole, 0.26 mmoles EDTA, 0.13 mmoles FeSO₄ • 7H₂O, 1.2 mmoles ascorbic acid, 1.2 ml H₂O₂ (30%) at pH 5.7 was applied to the column and washed with 0.05 M CH₃COOH at 2 °C. Fractions of 5 ml were collected and assayed for bound phosphate.

**Results**

Choosing imidazole as the substrate, in dilute solutions we observed the formation of a bound phosphate with a yield of 0.9% (based on the inorganic phosphate). Control experiments (Table I) show that omission of phosphate, imidazole, FeEDTA complex, or H₂O₂, suppressed bound phosphate formation. In contrast, omission of ascorbic acid only reduced the yield to 0.2% as expected regarding the role of ascorbic acid as a reductant of the Fe(III), and doubling the initial Fe(II) concentration increased the yield again to 0.5%.

EDTA was also not essential for the formation of bound phosphate since it could be replaced by oxalic acid or diethylene-triamine 1,1,4,7,7 pentaacetic acid. Replacing Fe(II) by Co(II), Mn(II), V(IV), and EDTA omitted, Cu(II) and EDTA yielded the same amount of bound phosphate as Fe(II).

Table I. Bound phosphate formation: The complete system contained the following components (μmol/ml).

<table>
<thead>
<tr>
<th>System</th>
<th>Yield of bound phosphate (μmol/ml) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Complete system</td>
<td>0.9</td>
</tr>
<tr>
<td>Imidazole omitted</td>
<td>0.9</td>
</tr>
<tr>
<td>Phosphate omitted</td>
<td>0.0</td>
</tr>
<tr>
<td>Fe(II)-EDTA omitted</td>
<td>0.0</td>
</tr>
<tr>
<td>H₂O₂ omitted</td>
<td>0.0</td>
</tr>
<tr>
<td>Ascorbic acid omitted</td>
<td>0.2</td>
</tr>
<tr>
<td>Ascorbic acid omitted and initial Fe(II)-EDTA concentration doubled</td>
<td>0.5</td>
</tr>
<tr>
<td>EDTA replaced by equimolar</td>
<td>0.8</td>
</tr>
<tr>
<td>oxalic acid</td>
<td>0.8</td>
</tr>
<tr>
<td>ethylene-triamine</td>
<td>0.9</td>
</tr>
<tr>
<td>1,1,4,7,7 pentaacetic acid</td>
<td>0.9</td>
</tr>
<tr>
<td>Fe(II) replaced by equimolar</td>
<td>0.0</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>0.0</td>
</tr>
<tr>
<td>Co(II)</td>
<td>0.0</td>
</tr>
<tr>
<td>V(IV)</td>
<td>0.0</td>
</tr>
<tr>
<td>Cu(II) and EDTA omitted</td>
<td>0.9</td>
</tr>
<tr>
<td>Imidazole replaced by equimolar</td>
<td>0.4</td>
</tr>
<tr>
<td>1-Methylimidazole</td>
<td>0.4</td>
</tr>
<tr>
<td>2-Methylimidazole</td>
<td>1.4</td>
</tr>
<tr>
<td>Hydroxymethylimidazole</td>
<td>0.3</td>
</tr>
<tr>
<td>Histamine</td>
<td>0.2</td>
</tr>
<tr>
<td>Pyrimidine</td>
<td>0.2</td>
</tr>
<tr>
<td>Imidazole replaced by 2-methylimidazole and 1 h bubbling with air instead of H₂O₂</td>
<td>0.7</td>
</tr>
</tbody>
</table>

V(IV) gave negative results; however, Cu(II) in the absence of EDTA (the complex with EDTA is not reduced by ascorbic acid) yielded the same amount of bound phosphate as Fe(II).

Replacing imidazole by other substrates, 1-methylimidazole, 2-methylimidazole, 4-hydroxy-
methylimidazole, histamine and pyrimidine gave positive results, 2-methylimidazole giving by far the best yields (Table I); the following substrates showed no detectable bound phosphate formation: imidazole-4-acrylic acid, histidine, adenine, xanthine, guanine, uracil, pyrazole, pyrazine, 1,2,4 triazole, acetonitril, imidazolidinithion, acetamide, cyanamid, dicyanamid, N,N dimethylformamid, oxamide, ethyleneurea.

Studying the time course of the reaction showed that bound phosphate attained a maximum immediately after the addition of hydrogen peroxide; then after a reaction period of maximally two minutes it is hydrolysed more or less rapidly, depending on the conditions. In order to characterize the hydrolytic behaviour, we adjusted the temperature and the pH of the solution to different values after two minutes of reaction time; Fig. 1 shows that both, increased temperature and pH, increase the rate of hydrolysis. The determination of the kinetic parameters from the initial slopes of the curves gave an observed rate constant of \( k = 1.7 \cdot 10^{-4} \text{ sec}^{-1} \) at 25 °C and pH = 0.1 and an activation energy of 20 kcal/mol between 25 and 35 °C for a first order rate of hydrolysis. The shape of the curve at pH 4.5 suggests the existence of at least two different phosphate species.

**Fig. 1.** Hydrolysis of bound phosphate(s): pH and temperature were adjusted to different values after two minutes of reaction time. The initial yield after this period was taken as 100%. Reaction conditions: A (see Table I).

**Fig. 2.** Effect of pH on bound phosphate formation: Substrates: 
- I = 2-methylimidazole; 
- II = imidazole; 
- III = 4-hydroxymethylimidazole; 
- IV = 1-methylimidazole; 
- V = pyrimidine. 
Reaction conditions: A (see Table I).
Fig. 3. Bound phosphate formation using 2-methylimidazole: Dependence of the yields on the concentrations of the constituents at 0 °C and pH = 6.0. With the exception of the component which is being varied the concentrations of the others are (in μmol/ml): inorganic phosphate 300; 2-methylimidazole 475; ascorbic acid 250; Fe(II) 25; EDTA 50; hydrogen peroxide 2000. Only in [(C) Fe(II) and EDTA are varied simultaneously at a constant ratio of 1:2.
Fig. 2 shows the dependence of the bound phosphate formation on the pH of the solution. As dependent on the pH of the solution at the beginning, we obtained characteristic maxima for each substrate.

Since 2-methylimidazole gave the greatest yields at pH 6 we used this substrate and higher concentrations of reagents for a study of the dependence of the yields on the various parameters. Fig. 3 shows the yields obtained from 2-methylimidazole as functions of the concentration of the components. In the case of inorganic phosphate and 2-methylimidazole as variables we observed linear dependence in small concentrations and saturation at higher concentrations. With Fe(II)-EDTA as variable, saturation was reached at very low concentrations, indicating the catalytic role of iron. Only in the case of ascorbic acid a maximum was observed. To understand the decrease of the yield at higher concentrations, in additional experiments we added ethanol or tert-butanol (250 μmoles per ml) and observed also a decrease of the yield (50%). Since alcohols are known radical trapping agents, the observed decrease of the yield at higher concentrations of ascorbic acid can be explained by trapping the OH radicals.

In other experiments we tried to replace hydrogen peroxide by O2. Using 2-methylimidazole as substrate, after one hour of bubbling air through the solution (without H2O2) easily detectable amounts of bound phosphate were obtained (Table I).

By chromatography of the reaction mixture on ion exchange columns two fractions of bound phosphate were obtained between elution volumes of 40–80 and 125–200 ml; inorganic phosphate remains absorbed on the column under these conditions. Work on isolation and identification of the phosphorylated product(s) is in progress.

Discussion

At this point little can be said about the mechanism of this unusual condensation reaction, except that energy rich radicals are probably involved in the trapping of inorganic phosphate. This may open a new aspect on biochemical processes like oxidative phosphorylation and photophosphorylation. Indeed, the molecular mechanism of both stays uncleared since the work of Lohmann on ATP [8]. Radical mechanisms in relation to these processes have been stressed rather exceptionally [9]. Usually two-electron processes have been considered in phosphorylation reactions [10] and general interest is presently focused on vectorial processes [11] in heterogenic systems, rather than on homogeneous reactions. Conspicuously enough, one electron processes are involved in the successful model systems of Wieland and Bäuerlein [12].

Only few cases of photophosphorylation models have been reported [9, 13]. However, such systems intrinsically imply the idea of one electron processes. Therefore, it appears significant that preliminary experiments with photosensitized oxidation of 2-methylimidazole under similar experimental conditions indeed gave rise [15] to bound phosphate formation. These results apparently justify the use of radical reactions as an experimentally more convenient model of photoreactions in the course of our “Hannover program” [3, 4, 14] – the experimental search for the first prebiological “Biods” [1-3], selforganizing systems which were capable to acquire and conserve the first bits of evolutionary information represented as steady states stabilized by distinct chemical feedback structures. Indeed, apart of feedback reactions, energy acquisition and transformation must be considered as the most important element of such mechanisms.

Note added in proof: A crystalline product, Schmp. 130 °C, has been isolated from the fraction eluted at 125-200 ml. Elemental analysis and NMR data are compatible with a O-monophosphoric acid ester of 4,5-dihydroxy-2-methyl-5′-imidazoline [15].