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Substrate and cofactor requirements of indoleamine 2,3-dioxygenase in interferon-gamma treated cells: Utilization of oxygen rather than superoxide

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Abstract

Much attention has been paid in initial biochemical studies on the ability of indoleamine 2,3-dioxygenase to use superoxide as substrate to cleave tryptophan to N-formyl kynurenine. This ability, however, is limited to the ferric form of the enzyme only, whereas the ferrous form requires oxygen rather than superoxide as substrate. As long as the enzyme is held in the ferrous form, high yield formation of product proceeds from the ferrous oxygen tryptophan ternary complex without the participation of superoxide. Enzyme assays in homogenates are carried out in presence of Methylene Blue, ascorbate and catalase. Ascorbate can be replaced by other reductants like e.g. tetrahydrobiopterin. Experiments with alteration of intracellular tetrahydrobiopterin concentrations in intact interferon-gamma treated cells clearly showed that tetrahydrobiopterin is not required for the indoleamine 2,3-dioxygenase reaction. In homogenates of interferon-gamma treated T-24 cells, substrates of xanthine oxidase did not stimulate the indoleamine 2,3-dioxygenase reaction, nor did allopurinol inhibit the reaction, nor did superoxide dismutase alter indoleamine 2,3-dioxygenase activity irrespective of the reductant used. From these experiments we concluded that molecular oxygen rather than superoxide is used in cell homogenates by indoleamine 2,3-dioxygenase to cleave L-tryptophan. A detailed analysis of available reports on oxygen and superoxide utilization by indoleamine 2,3-dioxygenase gives a comprehensive picture that the enzyme uses oxygen bound to the ferrous enzyme for cleavage of tryptophan, that the enzyme needs to be held by reductants in the ferrous state in enzyme incubations, and that superoxide is one of the reductants capable performing this reduction.

Keywords

indoleamine-pyrrole 2,3-dioxygenase; tetrahydrobiopterin; oxygen; superoxide; flavinmononucleotide; superoxide dismutase; tryptophan; interferon-gamma

Introduction

Indoleamine 2,3-dioxygenase (indoleamine-pyrrole 2,3-dioxygenase, EC-Number 1.13.11.42) was detected as a tryptophan cleaving enzyme widely distributed in the body and showing a wider substrate specificity than the classical liver tryptophan 2,3-dioxygenase (EC-Number 1.13.11.11, [1], reviewed in [2]). Indoleamine 2,3-dioxygenase catalyses the following reaction:

While tryptophan 2,3-dioxygenase is specific for L-tryptophan, indoleamine 2,3-dioxygenase does also cleave D-tryptophan and other indoleamines. L-Tryptophan is,

however, by far the preferred substrate of the enzyme. The enzyme cleaves tryptophan to N-formyl kynurenine by the incorporation of molecular oxygen, and thus initiates the oxidative degradation of tryptophan induced by proinflammatory cytokines like interferon-gamma (reviewed in [3]). Indoleamine 2,3-dioxygenase is thought to play a role in limiting parasite [4, 5] and viral [6] infections, in immunosuppression and pregnancy [7, 8], in tumour tolerance [9, 10] and in allergic immune responses [11].

The enzyme has recently been crystallized [12], and details how the reaction of the pyrrol moiety of tryptophan with molecular oxygen proceeds have been proposed on the basis of studies of the heme environment of recombinant human indoleamine 2,3-dioxygenase [13]: A proton is abstracted from the substrate by the ferrous oxygen tryptophan complex of the enzyme [13]. This mechanism is supported by the analysis of the crystal structure of the enzyme, which confirms that no protein group of indoleamine 2,3-dioxygenase is essential in dioxygen activation or proton abstraction [12].

Substrates of indoleamine 2,3-dioxygenase – oxygen or superoxide?

Literature survey

It appears to be widely accepted that indoleamine 2,3-dioxygenase uses oxygen as substrate. In enzyme databases such as BRENDA (<http://www.brenda.uni-koeln.de/>) or EXPASY (<http://www.expasy.org/enzyme/>) oxygen rather than superoxide is mentioned as substrate of the enzyme. O18 labelled oxygen has been shown to be incorporated into the reaction products even in presence of unlabelled superoxide [14].

Using purified enzyme, reactivity has been demonstrated also with superoxide [15, 16]. This reaction, however, is limited to the ferric form of the enzyme, which has to be reduced to the active ferrous form. Among other agents, superoxide is capable of reducing this ferric form to yield a ferrous oxygen complex [17], which then cleaves tryptophan to N-formyl kynurenine. When the enzyme is held in the ferrous state, molecular oxygen in form of the ferrous complex is the substrate for cleavage of tryptophan and no superoxide is required [18].

Enzyme assays are carried out in presence of Methylene Blue, ascorbate and catalase [1]. It was speculated that ascorbate and Methylene Blue in combination would supply the enzyme with superoxide [16]. Detailed examination [19] revealed, however, that at near maximal dioxygenase activity even 880 U/ml superoxide dismutase had no effect on indoleamine 2,3-dioxygenase activity. Anaerobic studies clearly showed that the reduced form of Methylene Blue (termed leuko Methylene Blue) can directly reduce ferric indoleamine 2,3-dioxygenase to the active ferrous form without requiring superoxide as intermediate [19]. In addition, tissue levels of superoxide might never reach the levels required by the ferric form of the enzyme [19], if it actually existed in tissues. In contrast to endotoxin treatment, hyperoxia did not lead to induction of indoleamine 2,3-dioxygenase in the lung of rats and mice, nor did indoleamine 2,3-dioxygenase induction protect the lung from oxidative damage [20].

To support a presumed involvement of superoxide in the indoleamine 2,3-dioxygenase reaction, homogenates of rabbit enterocytes have been incubated with inosine, with and without allopurinol, an inhibitor of xanthine oxidase [21]. Addition of inosine caused a marked increase in activity. This increase was blocked by allopurinol. Allopurinol, however, could not suppress the activity observed in the homogenate without inosine addition. Inactivation of superoxide dismutase by 5 mM diethyl dithio carbamate led to a concurrent increase in indoleamine 2,3-dioxygenase activity [21].

Investigations in homogenates of interferon-gamma treated T24 cells

We tested inhibition of indoleamine 2,3-dioxygenase and cofactor requirement in interferon-gamma stimulated T24 cells [22]. We found highest activity in presence of Methylene Blue (20 μ M), ascorbate (10 mM) and catalase (40000 U/ml). Ascorbate could be replaced by other reductants like NADPH or tetrahydrobiopterin, but Methylene Blue was additionally required for full activity: Kynurenine concentrations without Methylene Blue or with Methylene Blue without reductant were always below 1 μ M. Superoxide dismutase (100 U/ml), however, could not affect the activity of either reductant (Figure 1). Thus we assume that the reductants converted Methylene Blue to the leuko form, which then was able to hold the enzyme in the active ferrous state without requiring superoxide as intermediate. Superoxide dismutase had no effect on indoleamine 2,3-dioxygenase in homogenates even when up to 5000 U/ml were added to various amounts of cellular homogenate (Figure 2). We controlled that our superoxide dismutase used was active at the time of addition to the homogenate by the standard activity assay [23]. Diethyl dithio carbamate (5 mM) did not increase indoleamine 2,3-dioxygenase activity in interferon-gamma treated T24 cells, but was even inhibitory, similar to reports for the pure enzyme [21].

We then tried effects of the xanthine oxidase substrate inosine and the xanthine oxidase inhibitor allopurinol in our interferon-gamma treated homogenates. Neither inosine (100 μ M) nor allopurinol (100 μ M) had any effect on the activity of indoleamine 2,3-dioxygenase.

From these results we concluded that tryptophan cleavage by indoleamine 2,3-dioxygenase in homogenates of interferon-gamma treated cells occurred without the participation of superoxide anion by incorporation of molecular oxygen into L-tryptophan.

What is the physiological reductant in cells performing the role of reduced Methylene Blue in enzyme incubations?

No essential role for tetrahydrobiopterin in the indoleamine 2,3-dioxygenase reaction

It was suggested that tetrahydrobiopterin may play a role in holding the enzyme in the reduced form in vivo, thereby acting as cofactor [24, 25]. Tetrahydrobiopterin biosynthesis is induced in parallel to indoleamine 2,3-dioxygenase in human cells and cell lines [26]. To test a possible role of tetrahydrobiopterin in the indoleamine 2,3-dioxygenase reaction in intact cells, we manipulated intracellular tetrahydrobiopterin concentrations by 2,4 diamino 6-hydroxypyrimidine and by sepiapterin and monitored the tryptophan cleavage by intact interferon-gamma treated cells. Although tetrahydrobiopterin could be efficiently depleted in cells, cells produced exactly the same amount of kynurenine irrespective of their tetrahydrobiopterin content [27, 28]. These observations were independently confirmed by others [29].

While indoleamine 2,3-dioxygenase was insensitive to alteration in intracellular tetrahydrobiopterin levels, we found that nitric oxide synthase activity in intact cells did rise and fall parallel to changes with intracellular tetrahydrobiopterin [30]. Also these observations have been independently confirmed by others [31], reviewed in [32, 33].

FMNH₂ can replace leuko Methylene Blue in enzyme incubations

Using murine epididymal indoleamine 2,3-dioxygenase, FMN coupled with a regenerating system was found to lead to even higher activities than Methylene Blue and ascorbic acid, although the calculated K_m for FMNH₂ was 4 times higher than for Methylene Blue (13 μ M versus 3 μ M) [25, 34]. This stimulatory effect could only be partially inhibited by superoxide dismutase, although high activities were used. The authors discussed several

possibilities how the mechanism of the stimulation could be explained and also suggested that FMNH₂ might be required to reduce ferric to ferrous enzyme. It is not clear, however, whether FMNH₂ serves the same role also in vivo. The net reaction of tryptophan and oxygen to N-formyl kynurenine does not require the input of electrons. In this way indoleamine 2,3-dioxygenase differs from the pterin- and flavin- dependent mixed function monooxygenases. If the environment in the cells protected the enzyme from oxidation to the ferric form, no additional electron donor would be required.

Catalase is essential to protect the enzyme in incubation mixtures

As important as an electron donor to keep the enzyme in its ferrous state are high amounts of catalase to protect the enzyme from oxidative damage. With the purified murine epididymal enzyme, 4200 units/ml were required for optimal activity [25]. For interferon-gamma treated T24 cell homogenates, more than 1600 units/ml were required for maximal activity [22]. Hydrogen peroxide has recently been shown to inactivate the enzyme by oxidation of crucial cysteines [35].

Conclusions

Biochemical experiments clearly demonstrated that oxygen rather than superoxide is required by indoleamine 2,3-dioxygenase. In enzyme incubations, indoleamine 2,3-dioxygenase had to be kept in the active ferrous form. This could be achieved by reduced Methylene Blue, by FMNH₂ or by superoxide. Although in inflammation induction of indoleamine 2,3-dioxygenase was paralleled by an increase in NADPH oxidase capacity [36] and GTP cyclohydrolase I [26], indoleamine 2,3-dioxygenase activity in interferon-gamma treated cells required neither superoxide nor tetrahydrobiopterin. If the cellular environment protected indoleamine 2,3-dioxygenase from oxidation to the ferric form, no additional electron donor might be required for indoleamine 2,3-dioxygenase activity in intact tissues.

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Figure 1. Activity of indoleamine 2,3-dioxygenase in homogenates of interferon-gamma treated T24 cells

T24 cells were cultivated, treated with interferon-gamma and incubated for tryptophan cleavage as described [22]. Kynurenine formation was measured after incubation for 10 min at 37° C by HPLC. The following concentrations of compounds were added: Methylene Blue, 20 μM; ascorbate, 10 mM; NADPH, 800 μM; tetrahydrobiopterin (H₄biopterin), 400 μM; superoxide dismutase (SOD), 100 U/ml.

Figure 2. Dependence of indoleamine 2,3-dioxygenase activity on superoxide dismutase addition in homogenates of interferon-gamma treated T24 cells

T24 cells were cultivated, treated with interferon-gamma and incubated for tryptophan cleavage as described [22]. Kynurenine formation was measured after incubation for 10 min at 37° C by HPLC. a.: 149 μg cellular homogenate per ml added. b: 298 μg cellular homogenate per ml added.