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PHOTO-INDUCED COVALENT LABELLING OF MALATE DEHYDROGENASE BY QUERCETIN

Andrew P. Seddon and Kenneth T. Douglas*

Department of Chemistry, University of Essex, Colchester, CO4 3SQ, U.K.

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Summary: Quercetin powerfully inhibits malate dehydrogenase reversibly and cooperatively with 50% inhibition at 2.5μM at pH 7.50. Irradiation with light of wavelengths ≳350nm, of a mixture of malate dehydrogenase and quercetin leads to covalent inhibition whose extent is directly related to quercetin concentration, inversely related to enzyme concentration, and partially protected against by NADH. Prephotolysis of quercetin followed by incubation (in the dark) with malate dehydrogenase led to a time-dependent covalent inhibition.

Introduction

The soluble form of malate dehydrogenase is believed to participate in the cytoplasmic side of the malate shuttle, which transports malate (NADH equivalents) across the mitochondrial membrane. The mitochondrial form in addition to completing the malate shuttle is also required for the tricarboxylic acid cycle (1).

In view of the photolability (2,3) of quercetin (1) and its inhibition of tea plant malate dehydrogenase (4), we have investigated the quercetin inhibition of bovine heart malate dehydrogenase and now report that quercetin (3,3', 4', 5, 7-pentahydroxyflavone) can be used to covalently label this enzyme.

Materials and Methods

Quercetin (Aldrich Chemical Co.) was recrystallised from ethanol-water. Bovine heart malate dehydrogenase, oxaloacetic acid and NADH were from Sigma Chemical Co.. Spectrophotometric measurements were made at 25.0°C on a Carlo Erba Spectracomp 601 or Perkin Elmer SP8-100 Spectrophotometer. The assay for malate dehydrogenase (5) was conducted at pH 7.50 (0.1M KH_2PO_4 buffer) by following the decrease in absorbance at 340nm: the assay solution contained 0.1mM NADH and 0.245mM oxaloacetic acid (freshly prepared).

* Person to whom enquiries should be addressed.

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Photolyses were performed using an Applied Photophysics Carousel Quantum Yield Photoreactor with a 250w medium-pressure mercury lamp whose emitted light could be focussed on a quartz cell(s), thermostatted by means of a Dewar flask with a quartz window. Light wavelengths of >350nm were selected by means of a three-compartment quartz chemical filter cell (3x2cm. pathlength) containing appropriate preirradiated solutions (6). For photo-activated labelling, malate dehydrogenase (≲18 units/ml) was incubated with the appropriate species in the photoreactor cuvette at 4°C for the required time. Unreacted material was separated from protein by gel filtration (Sephadex G-25, 1.50 x 24cm), the eluted material (using pH 7.50, 0.1M KH_2PO_4 buffer) being assayed at several concentrations. This separation was arranged to provide reproducible dilutions of the applied protein sample, confirmed by independent G-25 passage of three solutions of enzyme. Eluted protein, collected in a single fraction, gave assay velocities proportional to the amount of enzyme present in the original stock soultions. This procedure was necessary as higher enzyme concentrations (suitable for A_{280} or dye-binding determination) could not be used for successful photoaffinity inhibition (vide infra).

Results

Quercetin inhibited bovine heart malate dehydrogenase at pH 7.50 in the dark, but with a complex, sigmoidal concentration dependence giving 50% inhibition at 2.5μM. This cooperative inhibition by quercetin was shown to be fully reversible by G-25 gel filtration after enzyme and quercetin had been incubated together for 25 mins. At 15cm. from the light source, with incident light of wavelengths >350nm, the enzyme (18 units/ml) lost 5.0% of its initial activity in 25 minutes (4°C, pH 7.50). Quercetin photolysed readily under similar conditions with concomitant destruction of the absorption at λ_{max} ∿380nm. The decrease in A_{380} with photolysis time fitted the first-order rate equation ($t_{\frac{1}{2}}$ = 9.9 min.) at 8.25μM quercetin. In 25 minutes the quercetin was 78% photolysed. At this pH, there were no significant hydrolytic problems for quercetin, although hydrolysis at higher pHs was measurable.

Photolysis of quercetin in the presence of malate dehydrogenase (≲18 units/ml) at pH 7.50 led to covalent inhibition (Figure 1). The experiment was run in triplicate using three different enzyme stock concentrations (18, 12 and 6 units/ml) each photolysed for 25 mins. in the presence of 16.3μM quercetin, 0.8% v/v purified dimethyl sulphoxide. This level of dimethyl

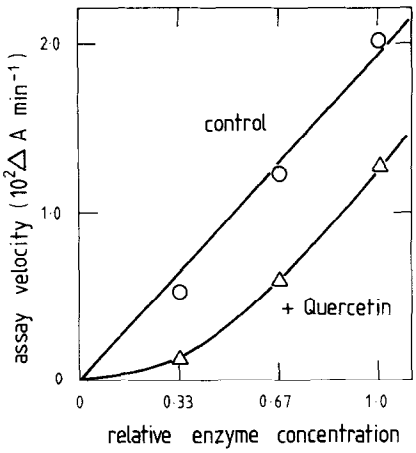


Figure 1
Plot of observed assay velocities for malate dehydrogenase activity at pH 7.50 for protein eluted from a G-25 column after 25 mins photolysis with incident radiation of wavelength >300nm at 4°C versus relative concentration of enzyme present in the initial incubation mixture prior to gel filtration (a relative concentration of 1.0 corresponds to an activity of 18 units/ml before gel filtration). The upper line is for control enzyme and the lower for enzyme in the presence of 16.3μM quercetin, both photoirradiated as described.

sulphoxide did not affect enzyme activity, although higher levels were inhibitory. After G-25 passage (calibrated conditions) the malate dehydrogenase activity of the eluted protein was assayed to obtain activities relative to a control system of enzyme (18, 12 and 6 units/ml) in the presenceof 0.8% dmso similarly treated (photolysis, G-25 passage). The degree of covalent inhibition was dependent on the amount of enzyme originally present (Figure 1): the percentage inhibition based on reisolated protein (after photolysis, G-25) decreased at higher initial enzyme concentrations. For the initial enzyme activities of 6,12 and 18 units/ml of this experiment, photo-covalent inhibitions of 84.0, 48.0 and 36.6% respectively, were obtained after 25 mins photoirradiation in the presence of 16.3μM quercetin. This phenomenon obviated working with higher enzyme concentrations and is presumably caused by some form of aggregation of the protein. Indeed, at 10-fold higher enzyme concentration than the greatest used for this data and with 8μM and 21μM quercetin, no covalent inhibition was obtained, even with prior gel-filtration (G-25) of the stock enzyme solution to remove potential protectors, of low

molecular weights. At 18 units/ml initial enzyme level the percentage inhibitions were 53.6, 46.0, 35.4 and 19.0% at 10, 10, 6.6 and 3.3μM quercetin, respectively. These covalent inhibition data were obtained at relatively high enzyme starting levels: higher percentage inactivations are possible by using lower enzyme concentrations. Photolysis of enzyme (12 units/ml) and quercetin (16.6μM) at 4°C (15 cm. from source) in the presence of 0.94mM NADH at pH 7.50 in 0.1M KH_2PO_4 buffer led to 21% covalent inhibition relative to a similarly-treated mixture of enzyme and NADH, in the absence of quercetin. In contrast, irreversible inhibition to an extent of 46% was observed in the absence of NADH indicating significant protection by NADH.

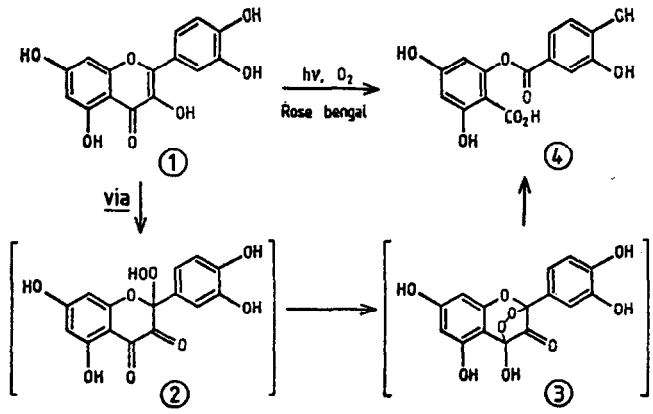
In any "photoaffinity labelling" experiment there exists the possibility that it is not a true photoaffinity labelling (i.e. photolysis of photolabile ligand while bound at the biological site leading to covalent labelling) but a secondary effect by photoproducts generated by photolysis of the photolabile ligand in free solution and not at the target biological site. These photoproducts may, in principle, attack the biological site concerned and covalently label it with a high or low degree of specificity. In the former case (tight binding of photoproducts) a photo-induced classical affinity-labelling experiment has resulted. Consequently, we investigated the effect of incubating unphotoirradiated enzyme (12 units/ml) with quercetin (16.6μM original concentration) which had been pre-photolysed under the chosen conditions above (25 mins.,15cm. from source, 4°C). Covalent inhibition by this solution of quercetin plus its photoproducts was observed, the degree of inhibition depending on the time of incubation of photolysed quercetin solution with the enzyme (with irreversible inhibitions of 21 and 53% for 25 and 60 min. incubations, respectively).

Discussion

The reversible inhibition we find prior to irradiation for malate dehydrogenase by quercetin is in agreement with other reports of flavone (4) and phenolic inhibitions (7). This irreversible enzyme inhibition under the

influence of light is novel, and complicated by a number of features, e.g. the decreased covalent photo-inhibition at higher enzyme concentrations. Perhaps more intriguing are the indications that the inhibition may not be a true photoaffinity experiment as pre-photolysed quercetin can lead to irreversible inhibition of malate dehydrogenase under conditions which the unphotolysed flavone does not. Further support of this lies in the time dependence of the extent of covalent inhibition by a prephotolysed solution of quercetin. Presumably an intermediate of reasonable stability (as it persists for at least 60 mins.) or a product derived from the photolysis of quercetin is the modifying agent. The rose bengal sensitised photo-oxygenation of quercetin has been postulated (2b) to involve hydroperoxide intermediates 2 and 3 (Scheme I) leading to an o-carboxyphenyl ester (4). Species 4 might be expected to acylate nucleophilic sites, especially with neighbouring group assistance from the CO_2H site (an aspirin analogue) and hydroperoxides 2 and 3 would be reactive. Prephotolysis of 1 could also lead to covalent inhibition of malate dehydrogenase by a mechanism as discussed above (i.e. via a hydroperoxide or acylating agent attack) with photolysis of quercetin in the presence of enzyme leading to covalent inhibition via a true photo-affinity process (e.g. intermediates 2 and 3, etc. being generated on the enzyme). Whichever mechanism operates the process is one of high specificity as high percentage irreversible inhibitions can be achieved either by prephotolysis of 1 (up to 53% inhibition) or cophotolysis of 1 plus enzyme ($\lesssim$84% inhibition), under conditions which were not fully-optimised for maximum covalent inhibition. Thus, it is likely that 1 is acting as an affinity label either directly or in pro-form. Quercetin on photoactivation has been reported to irreversibly inhibit glucose transport in human erythrocytes at pH 6, 20°C but few details are available for that study so that comparison is difficult (3).

The cooperative binding of quercetin might imply an allosteric binding site or cooperativity between subunits if the site used is close to or at



scheme 1

the active-site. Somewhat in favour of the latter is the protective effect of NADH on photolabelling reactions of quercetin, although this protection criterion is not clear cut for an oligomeric enzyme. The inhibition of malate dehydrogenase by p-bromophenol appears competitive with NAD^+ and malate in the forward reaction, it is actually mixed with respect to each substrate and in the reverse direction is competitive with NADH (7). Certain bioflavonoids, including quercetin, inhibit the glycolysis of a variety of tumour cells (8) and the growth of several cell lines (8) and possess marginal cytotoxicity in the P-388 lymphocytic leukaemia system in vitro (9). The high aerobic glycolysis of tumour cells leads to rapid lactate formation in tumour cells (9-12) which is blocked by low levels (16μg/ml) of quercetin (12). Efficient inhibition of malate dehydrogenase (e.g. 50% at 2.5μM) would lead to an increased level of cytosolic NADH which, if anything, would favour the formation of lactate in the pyruvate-lactate equilibrium. Thus, the malate dehydrogenase inhibition is unlikely to be directly causing the lowered lactate levels in tumour cells.

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