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Coenzyme B_{12}-dependent 2-methyleneglutarate mutase from *Clostridium barkeri*

Protection by the substrate from inactivation by light

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Partially purified 2-methyleneglutarate mutase from *Clostridium barkeri* was separated from 3-methylitaconate Δ-isomerase by treatment with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) followed by FPLC on the anion exchange column Mono Q in the presence of the detergent. When purified in the dark, the active mutase contained a corrinoid, most probably coenzyme B_{12}. The enzyme was inactivated by light at the same wavelength ($\lambda < 620$ nm) and rate as free coenzyme B_{12}. The rate was not influenced by oxygen or by temperature (0–37°C). Reactivation of up to 50% of the original activity was achieved by incubation with coenzyme B_{12} and dithiothreitol. The substrates 2-methyleneglutarate (up to 40 mM) or (*R*)-3-methylitaconate specifically protected the enzyme from inactivation by visible light. This effect was enhanced 3-fold by raising the temperature from 0°C to 37°C. The data indicate that during catalysis, the Co–C bond of the coenzyme is cleaved and cannot be affected any more by light.

Coenzyme B_{12}; Adenosylcobalamin; 2-Methyleneglutarate mutase; Visible light; *Clostridium barkeri*; Co–C bond cleavage

1. INTRODUCTION

2-Methyleneglutarate mutase catalyses the reversible carbon-carbon rearrangement of 2-methyleneglutarate to (*R*)-3-methylitaconate, a step in the fermentation of nicotinate by *Clostridium barkeri* [1–4]. The enzyme is strictly dependent on coenzyme B_{12} (adenosylcobalamin) which is involved in the transfer of the hydrogen atom during the rearrangement [5]. Studies with ^3H-labelled substrates as well as with ESR-spectroscopy showed that during catalysis of the related enzymes propanediol dehydratase and ethanolamine ammonia-lyase the Co–C bond of the coenzyme is cleaved homolytically whereby the active species, a 5′-deoxyadenosyl radical, is formed. The latter abstracts a hydrogen atom from the substrate yielding 5′-deoxyadenosine and a substrate radical which rearranges to a product radical. In the final step the hydrogen is donated back affording the product and regenerating the 5′-deoxyadenosyl radical (for reviews see [6,7]). Although it is generally accepted that this minimal mechanism is valid for all coenzyme B_{12}-dependent reactions, evidence for the group of enzymes catalysing carbon-carbon rearrangements is still weak [5,8].

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Here we report the partial purification of 2-methyleneglutarate mutase free of 3-methylitaconate Δ-isomerase at conditions under which a corrinoid, most probably coenzyme B_{12}, is retained on the active enzyme. In this state the enzyme is inactivated by visible light which is known to homolytically cleave the carbon–cobalt bond of the coenzyme [9]. Therefore it will be of interest to study the light sensitivity of the enzyme during the catalytic turnover in which a cleavage of this bond should be part of the mechanism.

2. MATERIALS AND METHODS

Sources and synthesis of 2-methyleneglutarate and (*R,S*)-3-methylitaconate were described earlier [3]. Coenzyme B_{12} and CHAPS were obtained from Sigma (Deisenhofen, Germany) and Biomol (Hamburg, Germany), respectively.

2.1. *Separation of 2-methyleneglutarate mutase and 3-methylitaconate Δ-isomerase*

2-Methyleneglutarate mutase (EC 5.4.99.4) and 3-methylitaconate Δ-isomerase (EC 5.3.3.6) from *C. barkeri* were purified together by chromatography on Q-Sepharose as described recently [4]. This as well as the consecutive steps were performed in a cold room in the dark or under red light. The active fractions (30 ml, 25 mg protein, mutase activity: 8 nkat/mg) were dialysed over night against 20 mM potassium phosphate, pH 7.4, containing 2 mM dithiothreitol. Then 6 mM CHAPS was added and the mixture was incubated at 23°C for 30 min. Afterwards it was pumped onto a Mono Q 10/10 column (Pharmacia, Freiburg, Germany) on which a linear NaCl gradient (0–400 mM, 200 ml, 1 ml/min) in the buffer used for dialysis containing 2 mM CHAPS was applied. 2-Methyleneglutarate mutase

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(25 nkat/mg) eluted at 300 mM NaCl, well separated from 3-methylitaconate Δ-isomerase (350 mM NaCl). Protein and the activities of the enzymes were determined as described recently [4]. However, in the assay of the mutase, dithiothreitol, coenzyme B_{12} and the preincubation were omitted.

2.3. *Experiments with light*

Monochromatic light was obtained by interference filters (band width 13 nm). The solutions (5 nkat enzyme or 10 μM coenzyme B_{12} in 100 mM potassium phosphate, pH 7.4; total volume 100 μl) were placed in the bottom of open 3 ml cuvettes (1 × 1 cm) and irradiated from the top at a constant power of 5 $J \cdot s^{-1} \cdot m^{-2}$ at 19°C. The response at 590 nm was linear from 4 to 7 $J \cdot s^{-1} \cdot m^{-2}$. Irradiations with polychromatic light were performed with an electric tungsten bulb (40 W) placed at a distance of 20 cm over 1 ml quartz cuvettes lying in a water-bath. They were closed with rubber stoppers and contained the same ingredients as above. Irradiations in the presence of substrate were performed under an atmosphere of nitrogen. One hour prior to the irradiation the substrate was added under anaerobic conditions. After the irradiations the cuvettes were used directly for the assay of the residual mutase activity.

3. RESULTS AND DISCUSSION

3.1. *Separation of 2-methyleneglutarate mutase from 3-methylitaconate Δ-isomerase*

The experiments described in this paper required more 2-methyleneglutarate mutase free of 3-methylitaconate Δ-isomerase than that obtained by the semi-preparative gel electrophoresis as reported recently [4]. But various chromatographic procedures were tested without success. Finally, incubation with the detergent CHAPS followed by FPLC on Mono Q resulted in the complete separation. When the same chromatography was performed in the absence of CHAPS both enzymes eluted together. Obviously, the enzymes form a complex stabilized by hydrophobic interactions. At this stage the mutase was not homogenous but could be used for the subsequent experiments. Contrary to earlier reports [4,5] the mutase was active without added coenzyme B_{12}. Addition of this compound to the assay stimulated by only 10%. Thus, the enzyme, purified in the dark or under red light, still contained coenzyme B_{12} which was also indicated by its absorption spectrum between 500–700 nm. In the earlier mutase preparations the coenzyme was destroyed by light during the purification procedure. It was not shown that the corrinoid of the enzyme was identical to coenzyme B_{12}. However, this appeared very likely, since the only corrinoid detected in *C. barkeri* was coenzyme B_{12} (E. Stupperich, personal communication).

3.2. *Inactivation of 2-methyleneglutarate mutase by light*

Irradiation of 2-methyleneglutarate mutase by an electric tungsten bulb rapidly destroyed the activity (Fig. 1). The rate was independent of temperature (0–37°C) and of the presence of oxygen. The same rate of inactivation was obtained with free coenzyme B_{12} under aerobic conditions. This was measured by the increase in absorption at 351 nm due to the formation of aquocobalamin [9]. The mutase was reactivated by coenzyme B_{12} up to 30–50% of the original activity. Higher values were never observed. Obviously, half of the enzyme was irreversibly destroyed, probably due to secondary reactions by free radicals generated by photolysis of the coenzyme [9]. Interestingly, the reactivation was independent of the presence of oxygen but absolutely dependent on 2 mM dithiothreitol. Also, no significant difference was observed either in the action spectra (Fig. 2) or in the absorbance spectra of mutase and free coenzyme B_{12} between 500–700 nm under aerobic conditions. As expected, both types of spectra matched each other. The enzyme as well as the coenzyme became inactivated only at wavelengths at which light was absorbed ($\lambda < 620$ nm). The data suggest that the coenzyme is bound to the enzyme without any conformational strain which is in contrast to observations with ethanolamine ammonia-lyase [6].

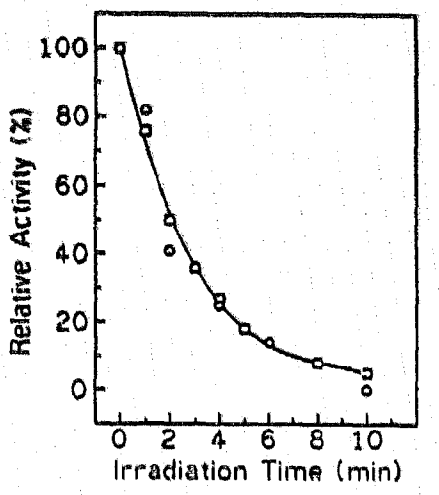

Fig. 1. Inactivation of free coenzyme B_{12} (circles) and 2-methyleneglutarate mutase (squares) by light from a tungsten bulb under aerobic conditions.

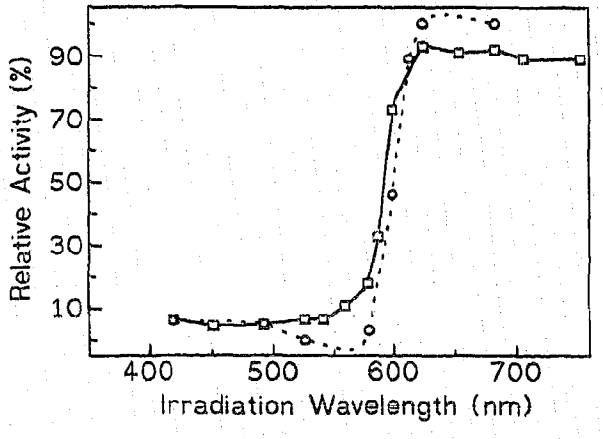

Fig. 2. Action spectra of free coenzyme B_{12} (circles) and 2-methyleneglutarate mutase (squares) of the inactivation by light under aerobic conditions.

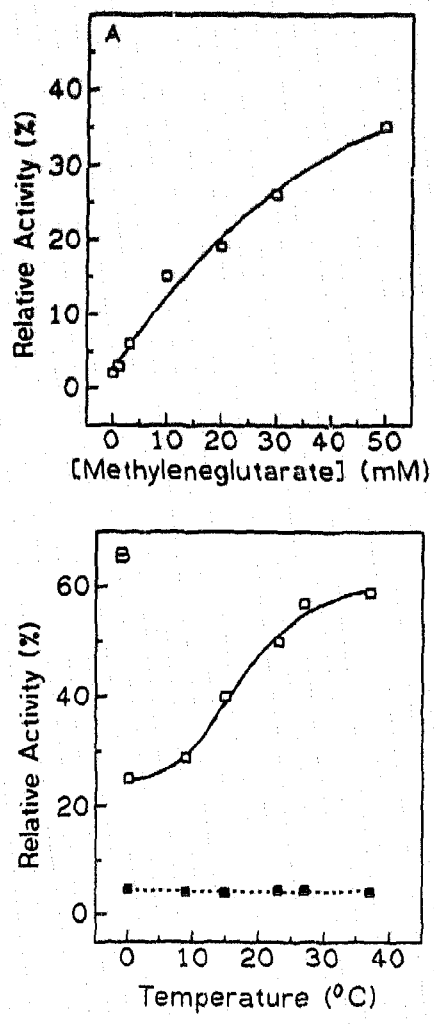

Fig. 3. Protection of purified 2-methyleneglutarate mutase by 2-methyleneglutarate from inactivation by light. (A) Dependence on the concentration of 2-methyleneglutarate at 0°C. (B) Dependence on the temperature in the presence of 30 mM 2-methyleneglutarate (open squares) or in the absence of substrate (closed squares). The activities were relative to that of an unirradiated enzyme sample.

3.3. *Protection by the substrates*

In order to measure the influence of the substrates 2-methyleneglutarate and 3-methylitaconate on the inactivation by light, the experiments had to be performed under anaerobic conditions. Although the mutase was stable under air, oxygen inactivated the enzyme during catalysis. The data of Fig. 3 show that 2-methyleneglutarate protected the mutase from inactivation by light under anaerobic conditions. For good protection saturating concentrations were required, about 10 times as high as the apparent K_m (4 mM [4]) (Fig. 3A). Under the conditions of the experiment the equilibrium between 2-methyleneglutarate and (*R*)-3-methylitaconate was reached [4]. Thus the latter compound should also be protective. This was tested with the racemic (*R,S*)-3-methylitaconate which was about 60% as active as 2-methyleneglutarate. That the reaction proceeded forward and backward during equilibrium was shown by the temperature dependence of the protection. Thus at higher temperatures at which the rates were higher (3-fold rate enhancement by raising the temperature from 13°C to 39°C) the activity recovered at 37°C was 3-fold higher than that at 0°C (Fig. 3B). In addition, the inhibitor succinate [5] as well as the non-inhibitory substrate analogues glutarate, (*R,S*)-2-methylglutarate or (*S*)-glutamate were unable to protect the enzyme. On the other hand, there was no effect of 2-methyleneglutarate on free coenzyme B_{12} indicating that the protection was not due to the absorbance of light by the dicarboxylic acid [4]. All these experiments showed that protection from light was only observed if the reaction proceeded.

These data can be explained by the minimal mechanism outlined in the introduction. Thereby the cobalt–carbon bond should be cleaved during the reaction. Thus this bond is not accessible any more for cleavage by light. Since no differences could be detected between free and bound coenzyme, it must have been the substrate which induced the cleavage of the cobalt–carbon bond into Co-II and a 5'-deoxyadenosyl radical. That these 2 reactive species were generated only in the presence of substrate was also indicated by the oxygen sensitivity of the enzyme during the reaction. An alternative mechanism in which the cobalt–carbon bond is cleaved by light and the substrate facilitates the recombination of both radicals in the dark cannot be excluded yet, but appears very unlikely. In summary this is the first demonstration that a coenzyme B_{12}-containing enzyme is protected by its substrate from inactivation by light.

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