Oxygen dependence of vitamin K-dependent carboxylase and vitamin K epoxidase

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Received 11 February 1986

A study of the oxygen requirements of the rat liver microsomal vitamin K-dependent carboxylase and vitamin K 2,3-epoxidase indicated that both enzymes had a K_m for O_2 in the range 60–80 μM. This value was not influenced by vitamin concentration, alterations in carboxylase substrate, Mn^{2+}, or dithiothreitol, and is consistent with the hypothesis that both activities are catalyzed by the same enzyme.

Vitamin K *Oxygen* *Carboxylation* *Epoxidation* *γ-Carboxyglutamic acid*

1. INTRODUCTION

The molecular mechanism involved in the vitamin K-dependent microsomal conversion of specific glutamyl residues to γ-carboxyglutamyl residues has not been established. The basic requirements of the in vitro system are O_2, CO_2, vitamin KH_2, and an exogenous peptide substrate or endogenous microsomal proteins [1]. Of these requirements, only the O_2 requirement has not been studied in detail. Early studies [2,3] established that oxygen was required for the carboxylation reaction, and a preliminary report of a lack of such a requirement has not been confirmed [4]. The only quantitative measurement of the O_2 requirement for carboxylation has been a report [5] that the vitamin K-dependent synthesis of prothrombin exhibited half-maximal activity at a pO_2 of about 10 mmHg.

A number of lines of evidence suggest that the microsomal formation of vitamin K 2,3-epoxide is associated with the carboxylation reaction. Both activities are located in the same tissues, concentrated in the rough endoplasmic reticulum, utilize the same forms of the vitamin, copurify, and are stimulated and inhibited by the same conditions [6]. Peptide substrates of the carboxylase stimulate epoxidase activity [7], and under conditions of saturating CO_2 a 1 : 1 ratio of CO_2 fixed to epoxide formed has been observed [8]. Growing evidence suggests a common enzymatic basis for the epoxidase and carboxylase activities [1], and in an attempt to clarify further the relationship between these reactions we have investigated their O_2 dependence. The influence of dithiothreitol (DTT) and $MnCl_2$ on O_2 uptake, carboxylation, and epoxidation was also investigated.

2. MATERIALS AND METHODS

Liver microsomal pellets were prepared from vitamin K-deficient rats as described [9]. The pellets were solubilized in SIK buffer (0.25 M sucrose, 0.025 M imidazole, 0.5 M KCl, pH 7.2) containing 1.0% Triton X-100, and debris removed by centrifugation at 100000 × g for 50 min. The solubilized microsomes were frozen, lyophilized, and stored at −20°C for up to 1 month without loss of vitamin K-dependent carboxylase or epoxidase activity.

Vitamin K-dependent carboxylase and vitamin K epoxidase activities were measured in duplicate at

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

17°C for 30 min in a shaker bath. Incubations contained 0.8 ml solubilized microsomes in a total volume of 1.1 ml consisting of a peptide substrate, 1 mM phenylmethylsulfonyl fluoride, 4 mM $NaHCO_3$, 20 μCi/ml of $NaH^{14}CO_3$ (60 mCi/mmol, Amersham-Searle) and 4–200 μg/ml of vitamin K hydroquinone added in 20 μl ethanol. Other additions are specified in the text. When feasible all solutions were boiled and degassed. After the nonvolatile components of the assay medium were added to the incubation mixture, the tubes were sealed with serum stoppers and O_2 removed from the system by purging with O_2-free N_2 for 1 h at 0°C. The deoxygenated tubes were then equilibrated with O_2/N_2 mixtures, and oxygen concentrations calculated from solubility curves or determined by an oxygen electrode. After addition of $NaHCO_3$ solution, reactions were initiated by the injection of vitamin KH_2. Carboxylation of Boc-Glu-Bz (Sigma, St. Louis, MO), Phe-Leu-Glu-Glu-Leu (Bachem, Torrance, CA) or endogenous protein substrates, and formation of vitamin K 2,3-epoxide were assayed as in [10–12]. Oxygen utilization in incubation mixtures was measured at 17°C in the reaction vessel of a Gilson oxygraph model KIC equipped with a water-jacketed Clark electrode which was standardized with air-saturated water.

3. RESULTS

The oxygen dependence of the vitamin-dependent carboxylation and epoxidation reactions are shown in fig.1. The similarity of O_2 dependence for the two reactions is evident from the saturation effect and similar apparent half-maximal activities. The K_m for O_2 for both of these reactions was measured under a number of conditions, and although variations in temperature and enzyme preparation did appear to have some influence, the values for both activities were similar over a wide range of carboxylation or epoxidation. Variation in the vitamin K concentration or alteration in the substrate for the enzyme (fig.2) did not have a large effect on the oxygen dependence of the carboxylation reaction, nor did additions of DTT or $MnCl_2$. Although there were variations in the measured values, these data indicated that the $K_{m,app}$ for O_2 is rather independent of a number of

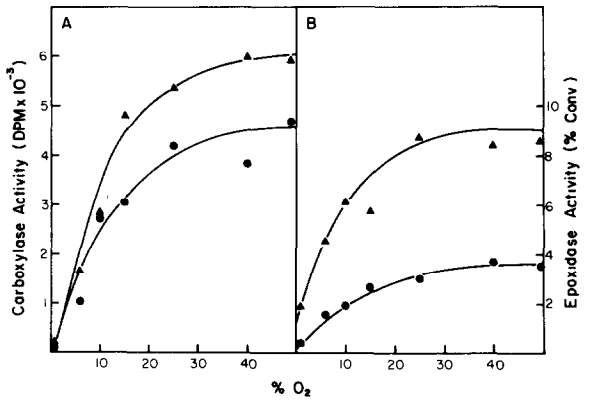

Fig.1. Effect of O_2 concentration on vitamin K-dependent carboxylation and epoxidation. Reactions were carried out at 17°C for 30 min as described in section 2 at a substrate concentration of 1 mM FLEEL. Vitamin KH_2 concentration was 100 μg/ml for carboxylation and 10 μg/ml for epoxidation. Peptide carboxylase activity (▲) is expressed as the amount of $^{14}CO_2$ fixed in a 200 μl aliquot of the incubation, and epoxidase activity (●) is expressed as the % of initial vitamin K converted to vitamin K epoxide. Microsomes were prepared from vitamin K-deficient rats (A) or from vitamin K-deficient rats given vitamin K to remove microsomal precursor protein (B).

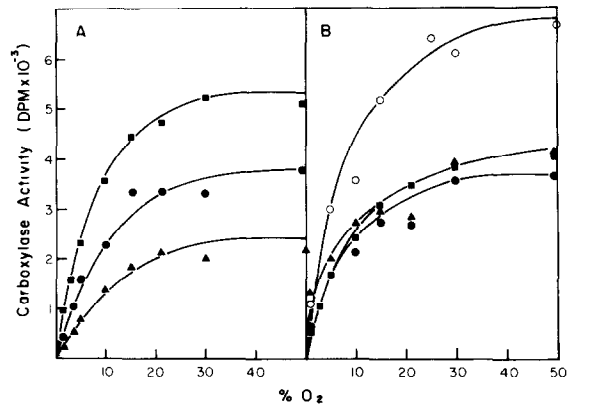

Fig.2. Effect of variations in vitamin K concentration and different substrates on the O_2 dependence of the vitamin K-dependent carboxylase. Incubations were carried out as described in section 2 and fig.1. Vitamin KH_2 concentration and $K_{m,app}$ for O_2 in panel A: ▲, 50 μg/ml (8.6); ●, 100 μg/ml (10.8); ■, 200 μg/ml (4.3). Substrates employed and $K_{m,app}$ for O_2 in panel B: ■, 10 mM Boc-Glu-Bz (5.5); ●, 1 mM FLEEL (2.8); ▲, 1 mM Boc-Glu-Glu-Leu-OMe (5.0); ○, endogenous microsomal proteins (4.0).

variables and was similar for both enzyme activities.

The average K_m value for O_2 determined in 6 assays incubated at 17°C was 5.1 ± 2.7% O_2 for pentapeptide carboxylation and 6.5 ± 1.7% O_2 for vitamin K epoxidation. Utilizing the O_2 concentrations of the incubation mixtures that were determined by the oxygraph, these values are equivalent to 62 ± 32 μM for carboxylation and 80 ± 22 μM for epoxidation.

The consumption of O_2 by the solubilized microsomal preparation was very slow in the absence of vitamin KH_2. Upon the addition of reduced vitamin, a marked increase in the rate of oxygen utilization which was dependent on the presence of microsomes and on the amount of added vitamin KH_2 was observed. However, as seen in fig.3, the boiled incubation mixture exhibits the same rate of O_2 uptake as the active microsomal solution. The presence of vitamin K quinone, the inhibitor Chloro-K, or a carboxylase substrate did not influence the rate of O_2 utilization or the total quantity of O_2 consumed (not shown). When $MnCl_2$ was included in the microsomal mixture, the rate of O_2 uptake was increased in both active and heat-denatured microsomes but not in SIK buffer. Interestingly, the addition of 1 mM DTT to the incubation mixture prevented this manganese stimulation of O_2 consumption.

Studies of O_2 consumption and carboxylation indicated that the cessation of carboxylation was associated with oxygen depletion which occurred rapidly in microsomal preparations supplemented with Mn^{2+}. Manganese stimulation of peptide carboxylase activity has been reported [13–15], and the data in fig.4 demonstrate that this stimulation is related to a 2-fold increase in the initial rate of the reaction and that the reported DTT stimulation of the preparation [3] is associated with a doubling of the period of linearity of the carboxylase reaction. The epoxidation of the vitamin (fig.4) exhibited the same time dependence as carboxylation and was stimulated in the same manner by manganese and DTT. The manganese effect was, however, limited to peptide substrate with vicinal glutamyl residues. Fig.5 demonstrates that manganese did not increase the initial velocity of either carboxylation or epoxidation associated with the simple glutamic acid derivative.

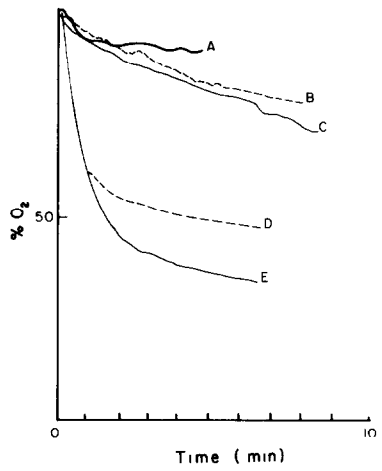

Fig.3. Oxygen consumption by microsomal carboxylase preparations. The initial concentration of O_2 in the oxygraph was 220 nmol/ml. At time 0, 100 μg/ml of vitamin KH_2 was added to (A) SIK buffer containing 1% Triton X-100, (B) a boiled and reoxygenated solubilized microsomal preparation, (C) an active solubilized microsomal preparation, (D) solubilized microsomes + 10 mM $MnCl_2$ followed by 1 mM DTT at 1 min, and (E) solubilized microsomes + 10 mM $MnCl_2$.

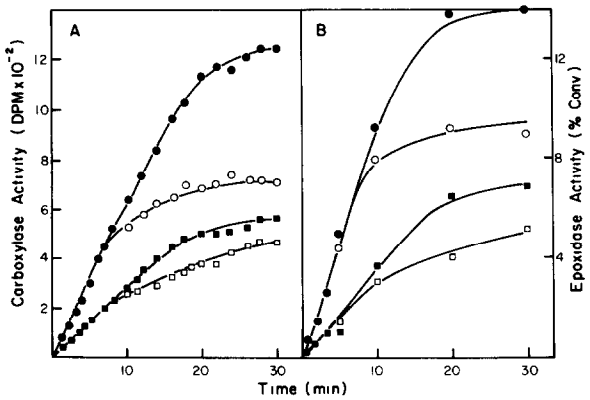

Fig.4. Effect of Mn^{2+} and DTT on carboxylation and epoxidation. Incubations were carried out as described in fig.1 and section 2 utilizing solubilized microsomal preparations without precursor proteins. The $MnCl_2$ and DTT concentrations were 10 and 1 mM, respectively. (A) Vitamin K-dependent peptide carboxylase activity: ●, $MnCl_2$ + DTT; ○, $MnCl_2$; ■, DTT; □, control. (B) Vitamin K epoxidase activity – conditions as in A.

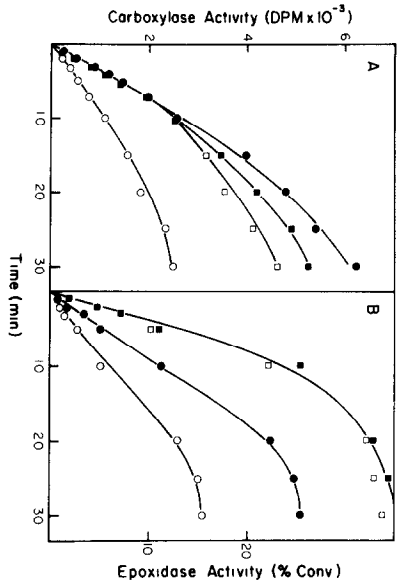

Fig.5. Effect of Mn^{2+} and substrate on carboxylation and epoxidation. Conditions as in fig.4 except for a vitamin KH_2 concentration of 4 μg/ml in the epoxidase experiments. (A) Vitamin K-dependent peptide carboxylase activity: ●, 1 mM FLEEL + 10 mM $MnCl_2$; ○, 1 mM FLEEL; ■, 10 mM Boc-Glu-Bz + 10 mM $MnCl_2$; □, 10 mM Boc-Glu-Bz.

4. DISCUSSION

Although details of the involvement of O_2 in the vitamin K-dependent carboxylation of glutamyl residues of microsomal proteins are not clear, there is substantial evidence [1] for a tight coupling between vitamin K-dependent carboxylase and epoxidase activities. This study provides further evidence for the association of these two enzyme activities by demonstrating that the oxygen dependences of vitamin K-dependent carboxylation and vitamin K epoxide formation are similar. The calculated K_m value for O_2 of 60–80 μM for both reactions is identical within experimental error and is somewhat higher than the K_m of 15 μM reported in [5] for formation of biologically active prothrombin. A more recent report [16] indicates a K_m for epoxidation in the same range as that reported here but did not allow a determination of a K_m for carboxylation. Oxygen dependence of other enzymes varies over a wide range of oxygen concentrations [17–19], and the similarity of K_m values for O_2 observed for both carboxylation and epoxidation would be unexpected if carboxylation and epoxidation were not coupled. Termination of carboxylase activity in these studies was associated with the complete oxidation of added vitamin KH_2, and this observation is consistent with the finding that compounds which stimulate or inhibit carboxylase activity may do so by altering the concentration of the reduced vitamin [20,21].

The present data suggest that the ability of manganese to stimulate carboxylation is specific to certain substrates. In contrast to [22], the carboxylation of Boc-Glu-Bz and the associated epoxidation of vitamin K were not stimulated by the presence of manganese, suggesting a role for manganese in the chelation of the carboxyl groups of the vicinal glutamyl residues of the peptide and/or a role in influencing the lipid-protein-detergent environment which allows the dianionic peptides more ready access to the enzyme [23,24]. Because the manganese stimulatory effect on carboxylase activity is substrate-specific, it is unlikely that the observed effect of manganese on the rate of oxidation of the reduced vitamin is coupled to its effect on carboxylase activity. The ability of DTT to prevent the manganese stimulation of O_2 uptake and the associated oxidation of vitamin KH_2 is noteworthy because of the reported ability of DTT to stimulate carboxylase activity [3] which has been postulated to be associated with an ability to maintain the reduced vitamin concentration [25]. The reported inconsistencies [3,25–27] in DTT stimulation of carboxylase may be due to differences in incubation times and initial vitamin concentrations. At saturating vitamin concentrations and shorter assay times, the presence of DTT would not influence the linearity of the carboxylase reaction, and no DTT effect would be observed.

ACKNOWLEDGEMENTS

This research was supported by the College of Agricultural and Life Sciences of the University of Wisconsin-Madison and in part by grants AM-14881 and DE-07031 of the National Institutes of Health, Bethesda, MD.

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