Biochem. J. (1974) **143**, 149–157
Printed in Great Britain

Association of Xanthine Oxidase with the Bovine Milk-Fat-Globule Membrane

CATALYTIC PROPERTIES OF THE FREE AND MEMBRANE-BOUND ENZYME

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(*Received* 10 *June* 1974)

1. The catalytic properties of xanthine oxidase in bovine milk (EC 1.2.3.2) are dependent on the state of the enzyme, i.e. whether free or bound to the fat-globule membrane. Oxidase activity of the membrane-bound enzyme towards NADH is enhanced relative to that towards xanthine. This reflects a change in the relative K_m values and enables the ratio of xanthine to NADH oxidase activities (X/N) to be used as a parameter for the relative amounts of free and membrane-bound xanthine oxidase in milk fractions. 2. Chromatography of buttermilk on Sepharose 2B yielded an excluded fraction, BM_1, with xanthine oxidase activity. The remaining xanthine oxidase activity was eluted as a single broad peak. This was further resolved on Sephadex G-200 into an excluded fraction, BM_2, and free xanthine oxidase. Fractions BM_1 and BM_2 had X/N values in the range 45–65, which is characteristic of membrane-bound xanthine oxidase. Purified xanthine oxidase has a mean X/N value of 110. 3. Addition of fraction BM_1, heated to remove associated enzyme activities, to purified xanthine oxidase progressively enhanced its NADH oxidase activity to a value where its X/N value was characteristic of membrane-bound xanthine oxidase. This was shown to be due to binding of free enzyme to heated fraction BM_1. The binding constant and stoicheiometry were determined. 4. Proteolytic digestion of fraction BM_1 liberated free xanthine oxidase from the fat-globule membrane with a corresponding alteration in X/N value.

Xanthine oxidase (EC 1.2.3.2) from bovine milk has been highly purified and extensively studied (Hart *et al.*, 1970; Edmonson *et al.*, 1972). Its composition and mechanism of action are now relatively well understood (Bray, 1963; Massey *et al.*, 1969). In milk much of this enzyme is known to be associated with the fat-globule membrane (Morton, 1954; Zittle *et al.*, 1956). This membrane is considered to be directly derived from the plasma membrane (Keenan *et al.*, 1971) which envelops the fat-globule as it is released from the secretory cell of the mammary gland.

The nature of the association of xanthine oxidase with the fat-globule membrane and its effects on the properties of the enzyme have received little attention. Ready availability of the purified enzyme and of large quantities of the fat-globule membrane makes this system a useful one in which to study enzyme–membrane interactions.

In this paper we report a procedure for isolating fat-globule membrane material and some differences in the catalytic properties of xanthine oxidase when free and when associated with the fat-globule membrane. A preliminary report of this work was presented at the 8th FEBS Meeting, Amsterdam, August 1972 (Abstract no. 155).

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Experimental

Methods

Preparation of buttermilk. Cooled milk, pooled from several mixed herds, was warmed to 40°C. Cream was separated with an Alfa Laval AE Farm Separator, adjusted to give cream of 40% (v/v) fat content. The cream was cooled to 8–10°C and churned by shaking. The aqueous phase (buttermilk) was decanted and centrifuged at 3500 *g* for 10 min to remove remaining free fat as a surface layer and casein micelles as a pellet. The buttermilk was stored at 4°C in the presence of added 0.02% (w/v) sodium azide until required. The xanthine oxidase activity remained constant for at least 2 months.

Enzyme assays. Xanthine (or hypoxanthine) oxidase activity was assayed spectrophotometrically (Kalckar, 1947; Avis *et al.*, 1955). The reaction mixture (final volume 2.5 ml) contained 250 nmol of xanthine (or hypoxanthine), 2.5 nmol of EDTA and enzyme in 50 mM-sodium pyrophosphate buffer (adjusted to pH 7.0 with 1 M-HCl). The reaction was started by the addition of enzyme and the increase

in E_{290} measured. NADH oxidase activity was assayed by following the decrease in E_{340} by using the same reaction mixture with 235nmol of NADH replacing xanthine. Dehydrogenase activities were assayed under the same conditions with the addition of 25nmol of 2,3,5-triphenyltetrazolium chloride by measuring increase in E_{255}. Alkaline phosphatase activity was assayed by following the hydrolysis of *p*-nitrophenyl phosphate as described by Bessey *et al.* (1946). All assays were carried out in a cuvette (10mm light-path) at 37°C in a Unicam SP 500 spectrophotometer with a direct read-out facility coupled to an SP 45 scale-expansion unit. Initial rates were taken from the linear portion of the progress curves and were proportional to enzyme concentration; 1 unit of xanthine oxidase activity is defined as that amount of enzyme causing a change in absorbance of 1.0 per min at 290nm with xanthine as substrate and oxygen as electron acceptor under the conditions described above; 1 unit of NADH oxidase activity is defined similarly in terms of a change in absorbance at 340nm of 1.0 per min with NADH as substrate.

Chemical analyses. Protein was estimated by the method of Lowry *et al.* (1951) with dry bovine serum albumin as standard.

Total lipid was determined gravimetrically. Freeze-dried samples were extracted with 2×10ml of hot ethanol–ether (3:1, v/v) followed by 2×10ml of chloroform–methanol (2:1, v/v). The extracts were combined and evaporated under vacuum to constant weight.

Lipid phosphorus was determined from the above extract which was wet ashed with H_2SO_4 and $HClO_4$ until clear. Samples, neutralized with NaOH, were assayed for phosphorus by the method of Chen *et al.* (1956). Estimates of phospholipid content are based on a mean molecular weight for phospholipids of the milk-fat-globule membrane of 800 (O'Mahoney, 1970).

Materials

Purified xanthine oxidase (from milk) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., Sepharose 2B and Sephadex C-200 from Pharmacia (G.B.) Ltd. London, W.5, U.K., *p*-nitrophenyl phosphate from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. and NADH (grade II) from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Bovine serum albumin (fraction V), pancreatin and other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

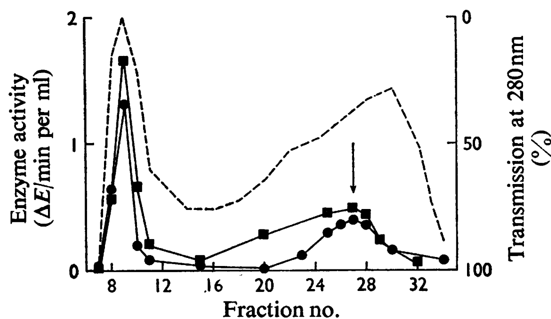

Fig. 1. *Fractionation of membrane-bound xanthine oxidase from buttermilk on Sepharose 2B*

A sample (15ml) of buttermilk, prepared as described in the text, was applied to a column (2.5cm×40cm) of Sepharose 2B (V_0 = 45ml) equilibrated with 0.1M-sodium pyrophosphate buffer, pH7.0. The column was eluted at 10ml/h with the same buffer at 4°C. Fractions (5ml) were collected. Percentage transmission at 280nm (----) was monitored with an LKB Uvicord. Xanthine oxidase (●) and alkaline phosphatase (■) activities were assayed as described under 'Methods'. Fractions 8–10 were combined and designated fraction BM_1.

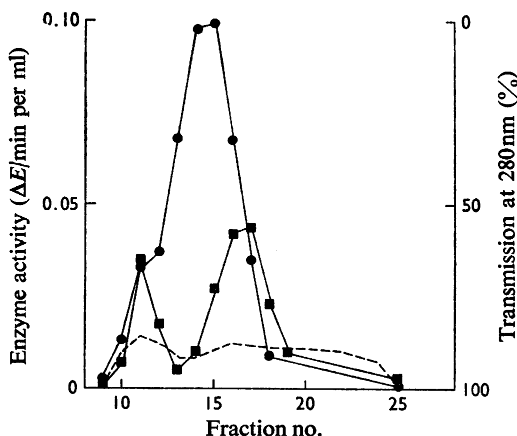

Fig. 2. *Fractionation of free and membrane-bound xanthine oxidase from buttermilk on Sephadex G-200*

The fraction (5ml) indicated by an arrow in Fig. 1 was applied to a column (2.5cm×41cm) of Sephadex G-200 (V_0 = 55ml) which was equilibrated and eluted as described in Fig. 1. Fractions (5ml) were collected and percentage transmission at 280nm (----), xanthine oxidase (●) and alkaline phosphatase (■) activities measured.

Results

Fractionation of buttermilk

The xanthine oxidase activity of buttermilk was separated into two fractions by column chromatography on Sepharose 2B (Fig. 1). The first fraction, designated fraction BM_1, which was eluted in the

Table 1. *Chemical composition of fractions BM_1 and BM_2*

Protein, total lipid and phospholipid content was determined as described under 'Methods'. Percentages were calculated assuming protein+total lipid = 100%.

	Protein (%)	Total lipid (%)	Phospholipid (% of total lipid)
Fraction BM_1	50.1–54.8	45.2–49.9	19.7–31.8
Fraction BM_2	72.5–81.9	18.1–27.5	15.3–17.9
Characteristic values for milk-fat-globule membrane (Brunner, 1965)	30–60	40–70	20.4–28.7

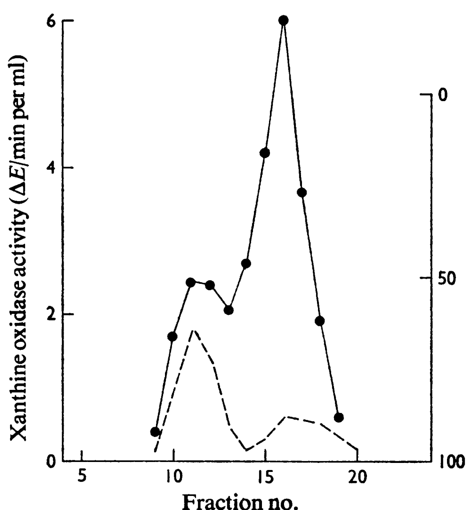

Fig. 3. *Fractionation of free and membrane-bound xanthine oxidase from skimmed milk on Sephadex G-200*

A sample (15ml) of skimmed milk, treated as described in the text was applied to a column (2.5cm×41cm) of Sephadex G-200 (V_0 = 55ml) which was equilibrated and eluted as described in Fig. 1. Fractions (5ml) were collected and percentage transmission at 280nm (----) and xanthine oxidase activity (●) measured.

void volume, was a cloudy solution which contained 42% of the applied xanthine oxidase activity and 18% of the applied alkaline phosphatase activity. Most of the remaining xanthine oxidase (46% of the applied activity) was eluted as a further single peak along with 26% of the applied alkaline phosphatase activity. (The apparently low recovery of alkaline phosphatase was due to inhibition by pyrophosphate in the elution buffer.) The enzyme activity of the most active fraction of the second peak (indicated by an arrow in Fig. 1) was resolved by chromatography on Sephadex G-200 (Fig. 2). The fraction eluted in the void volume, designated fraction BM_2, contained 8% of the xanthine oxidase activity and 10% of the alkaline phosphatase activity applied to the column. Two further fractions were eluted which contained largely xanthine oxidase (83% of the activity applied) and alkaline phosphatase (25% of the activity applied) respectively. Typical compositions of fractions BM_1 and BM_2 are presented in Table 1.

Isolation of free xanthine oxidase from skimmed milk

A widely used method for isolating xanthine oxidase from milk involves proteolytic digestion with pancreatin at an early stage in the purification procedure (Gilbert & Bergel, 1964). To determine whether this treatment alters the properties of xanthine oxidase compared with those of the free enzyme existing in milk, free xanthine oxidase was isolated from skimmed milk.

Skimmed milk, remaining after separation of cream from whole milk (see under 'Methods'), at 4°C was brought to pH4.0 by addition of 1M-HCl. The resulting precipitate (of casein) was separated by centrifugation and the supernatant immediately fractionated on a column of Sephadex G-200 (Fig. 3). (Less than 10% of the xanthine oxidase activity was lost during storage of the above supernatant at 4°C for 15 days.) The first peak, which was eluted in the void volume, contained 30% of the applied xanthine oxidase activity together with some alkaline phosphatase activity. The second peak, which contained 65% of the applied xanthine oxidase activity, was virtually free of alkaline phosphatase activity. A sample of purified xanthine oxidase was eluted from Sephadex G-200 as a single peak in the same position as the second peak from the acidified skimmed-milk supernatant.

Specificity of free and membrane-bound xanthine oxidase

The presence of membrane-bound xanthine oxidase in buttermilk is indicated by the appearance of xanthine oxidase activity in lipoprotein material excluded from Sepharose 2B (excludes particles >4×10^7daltons). Differences in substrate specificity between fraction BM_1 and purified xanthine oxidase might provide a convenient kinetic parameter for measuring the relative amounts

Table 2. *Activity ratios of various substrate–electron acceptor couples with free and membrane-bound xanthine oxidase*

Activities of purified xanthine oxidase and fraction BM_1 were determined by using xanthine, hypoxanthine or NADH as substrate and oxygen or 2,3,5-triphenyltetrazolium chloride as electron acceptor as described under 'Methods'. The following abbreviation is used: tetrazolium = 2,3,5-triphenyltetrazolium chloride.

	Ratio of enzyme activities	
Substrate–electron acceptor couple	Purified xanthine oxidase	Fraction BM_1
Xanthine–oxygen/xanthine–tetrazolium	3.8	3.9
Xanthine–oxygen/hypoxanthine–tetrazolium	3.6	3.8
Xanthine–oxygen/hypoxanthine–oxygen	1.8	3.1
Xanthine–oxygen/NADH–oxygen	110.0	52.0
Hypoxanthine–oxygen/xanthine–tetrazolium	2.1	1.3
Hypoxanthine–oxygen/hypoxanthine–tetrazolium	2.0	1.3
Hypoxanthine–tetrazolium/xanthine–tetrazolium	1.1	1.0

Table 3. *Kinetic parameters for NADH and xanthine as substrates of free and membrane-bound xanthine oxidase (O_2 as electron acceptor)*

Enzyme activities were assayed as described under 'Methods'. Velocities were measured (in duplicate) at five or six different substrate concentrations. Values of apparent K_m and V (±S.E.M.) were calculated from plots of $1/v$ versus $1/s$ by the method of least squares with an ICL 450 computer. No weighting was used.

	K_m for xanthine (μM)	K_m for NADH (μM)	$\frac{V_{xanthine}\ (E_{290}/\text{min})}{V_{NADH}\ (E_{340}/\text{min})}$
Purified xanthine oxidase	0.291±0.009	121.7±5.1	62.07±2.39
Fraction BM_1	0.541±0.030	40.0±1.4	53.74±6.93

of free and membrane-bound xanthine oxidase in milk fractions.

Oxidase and dehydrogenase activities of fraction BM_1 and purified xanthine oxidase were determined with xanthine, hypoxanthine or NADH as electron-donor substrate, and oxygen or 2,3,5-triphenyltetrazolium chloride as electron acceptor. The specificities of the two forms of the enzyme are compared in terms of the ratios of the activities of each form towards various substrate–electron acceptor couples. The results (Table 2) show that the ratio of the activities, xanthine–oxygen/NADH–oxygen, designated X/N, is the most sensitive for distinguishing free and membrane-bound xanthine oxidase.

The kinetic parameters of free and membrane-bound xanthine oxidase for NADH and for xanthine are presented in Table 3. Both forms of the enzyme exhibited normal Michaelis–Menten kinetics with either substrate. Comparison of the apparent K_m values shows that membrane-bound xanthine oxidase has a greater affinity for NADH than does the free enzyme. With xanthine as substrate the reverse is true.

The absolute amount of enzyme present in a membrane is difficult to determine without reference to its catalytic activity. This complicates interpretation of the comparison of V values of membrane-bound and soluble forms of the enzyme for a given substrate. By using two substrates, however, it is possible to compare the ratios of their V values and interpret these ratios in terms of relative turnover numbers of each form of the enzyme. Comparison of the ratio $V_{xanthine}/V_{NADH}$ of fraction BM_1 and of purified xanthine oxidase (Table 3) reveals no significant difference ($P<0.001$). This indicates that the ratio of the turnover numbers of xanthine oxidase for these two substrates is not affected by association with the fat-globule membrane.

Under the assay conditions used, the concentration of xanthine is about one hundred times the apparent K_m of either fraction BM_1 or the purified enzyme for this substrate, whereas the concentration of NADH is of the same order of magnitude as its apparent K_m values. The values of X/N of purified xanthine oxidase and of fraction BM_1 therefore reflect the differing affinities of the free and membrane-bound enzyme for NADH.

Fractions BM_1 and BM_2 from buttermilk (Figs. 1 and 2) and the skimmed-milk fraction excluded from Sephadex G-200 (Fig. 3) all had X/N values in the range 45–65. The X/N values of purified xanthine oxidase and of free xanthine oxidase fractionated from either skimmed milk or buttermilk were consistently within the range 95–130.

Binding of xanthine oxidase to heat-treated membrane preparations

The different X/N values of free and membrane-bound preparations of xanthine oxidase are due to higher NADH oxidase activities of the membrane fractions. The enhanced NADH oxidase activity might be attributable either to an alteration of the catalytic properties of bound xanthine oxidase, or simply to the presence of another NADH oxidase enzyme associated with the membrane. To provide evidence on this point, binding characteristics were studied on free xanthine oxidase and a membrane preparation devoid of associated enzyme activities.

Fraction BM_1 was heated for 10min in boiling water to inactivate bound enzymes. The resulting suspension was free of detectable alkaline phosphatase, NADH oxidase, and xanthine oxidase activities. Portions of the heat-treated membrane preparation were added to a sample of purified xanthine oxidase in a cuvette containing the NADH oxidase assay mixture (see under 'Methods'). The rate of NADH oxidation was measured after each addition by following the decrease in absorbance at 340nm. NADH oxidase activity increased progressively with each addition to a plateau (Fig. 4). A similar experiment, substituting xanthine for NADH, showed that oxidase activity towards xanthine remained unchanged. Thus titration of free xanthine oxidase with heated fraction BM_1 effectively changes the X/N value from that characteristic of the free enzyme (X/N = 110) to that characteristic of the membrane-bound enzyme (X/N = 50) (Fig. 4).

Identical results were obtained with free xanthine oxidase from skimmed milk in place of the purified enzyme. The skimmed-milk fraction, excluded from Sephadex G-200 (Fig. 3), could substitute for fraction BM_1 to give a similar titration curve. The enhancement of NADH oxidase activity of free xanthine oxidase was not observed on addition of heated fraction BM_2, casein (up to 20mg/xanthine oxidase unit) or a mixture of protein, lipid and phospholipid in the proportions found in fraction BM_1 (1.2mg of bovine serum albumin, 0.6mg of glycerol tripalmitate and 0.6mg of DL-phosphatidylcholine per ml, homogenized in 0.1M-sodium pyrophosphate buffer, pH7.0).

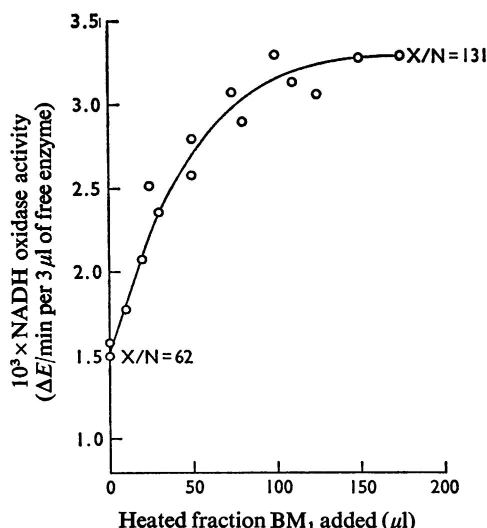

Fig. 4. *Effect of heated fraction BM_1 on NADH oxidase activity of free xanthine oxidase*

Purified xanthine oxidase (0.18 xanthine oxidase unit) was placed in a reaction cuvette containing the NADH oxidase assay mixture (see under 'Methods'). Increments of heated fraction BM_1 were added and the NADH oxidase activity was measured after each addition. Activities were measured as described under 'Methods' and were corrected for volume changes occurring in the reaction cuvette. Progress curves remained linear throughout the experiment. X/N values were calculated from the NADH oxidase activities and the results of a parallel experiment in which xanthine oxidase activity was measured.

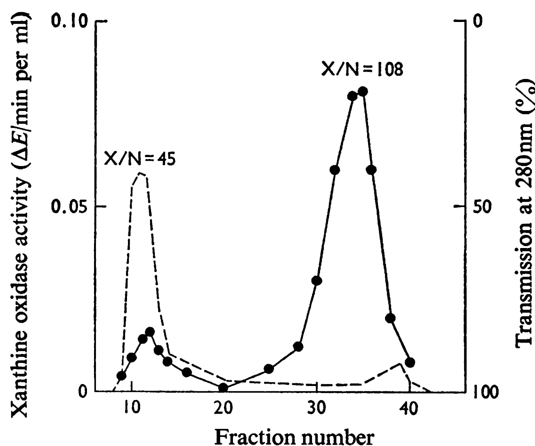

Fig. 5. *Fractionation of a mixture of heated fraction BM_1 and free xanthine oxidase (X/N = 62) on Sepharose 2B*

A mixture of purified xanthine oxidase (3.26 xanthine oxidase units) and heated fraction BM_1 (7.32 xanthine oxidase units before heating), prepared as described in Fig. 4 and having an X/N value of 62, was applied to a column (2.5cm×40cm) of Sepharose 2B (V_0 = 50ml) which was equilibrated and eluted as described in Fig. 1. Fractions (5ml) were collected and percentage transmission at 280nm (----) and xanthine oxidase activity (●) measured. X/N values were calculated from NADH oxidase activities of the fractions in each peak having the highest xanthine oxidase activity.

Chromatography on Sepharose 2B of a mixture of purified xanthine oxidase and heated fraction BM_1, having an X/N value of 62, eluted 20% of the applied xanthine oxidase in the void volume (Fig. 5). The X/N value of this remaining fraction was 45, characteristic of membrane-bound enzyme. The remaining activity was eluted in a single peak (X/N = 108).

Determination of binding parameters

These results suggest that membrane-bound enzyme is present in mixtures of heated fraction BM_1 and purified xanthine oxidase. By using the enhancement of NADH oxidase activity as an indicator of enzyme–membrane association, the binding parameters were determined from titration of heated fraction BM_1 with free xanthine oxidase.

Assuming dynamic equilibrium between enzyme and membrane binding site,

$$EM \rightleftharpoons E+M,$$

the intrinsic dissociation constant, K, can be expressed as

$$K=\frac{(E_f)n(M)}{E_b} \quad (1)$$

where E_f = concentration of added xanthine oxidase unbound (in xanthine oxidase units), E_b = concentration of added xanthine oxidase bound (in xanthine oxidase units), n = number of enzyme units bound to the membrane per xanthine oxidase unit originally present on fraction BM_1 (i.e. before heating), M = concentration of unoccupied binding sites, in terms of xanthine oxidase units originally present on fraction BM_1 (i.e. before heating). The experimentally determined quantities are M_t = total concentration of binding sites originally present on fraction BM_1 (as xanthine oxidase units), A = observed increased concentration of NADH oxidase activity of added xanthine oxidase (as NADH oxidase units), E_t = total concentration of added enzyme (as xanthine oxidase units). Then nM = concentration of unoccupied sites actually present and nM_t = total concentration of xanthine oxidase binding sites on heated fraction BM_1. Assuming that enhancement of NADH oxidase activity is due to enzyme–membrane association, and taking 50 as the mean X/N value for membrane-bound xanthine oxidase, the concentration of bound enzyme can be calculated from the increase in NADH oxidase activity observed when free xanthine oxidase is added to the membrane preparation. The observation that oxidase activity towards xanthine is unaffected by heated fraction BM_1, allows the use of the conservation equation for enzyme to calculate E_f.

Thus

$$E_b = 50A$$

and

$$E_f = E_t - 50A$$

The concentration of unoccupied sites can be calculated from the conservation equation for binding sites

$$nM_t = nM + E_b$$

Substituting in eqn. (1)

$$K=\frac{(E_t-50A)(nM_t-50A)}{50A}$$

or

$$K=\frac{nM_t E_f}{E_b}-E_f$$

If the assumptions made are correct, a plot of E_f/E_b against E_f should be linear with slope = $1/nM_t$ and x axis intercept = $-K$. Such a plot of the data from titration of heated fraction BM_1 with purified xanthine oxidase is shown in Fig. 6. The value of the dissociation constant, K, calculated from this plot is 0.115 xanthine oxidase unit per ml; n = 1.60 xanthine oxidase units bound per unit originally present in fraction BM_1 (before heat treatment).

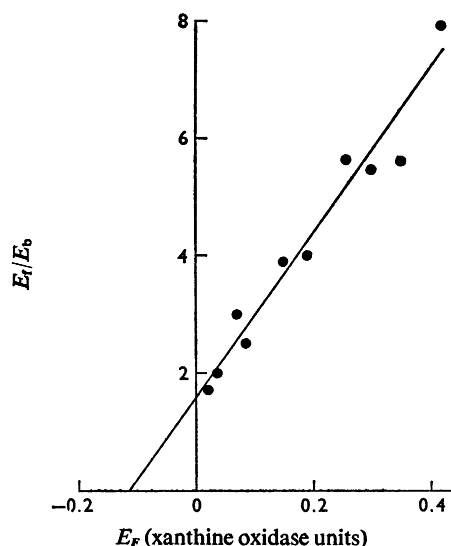

Fig. 6. *Binding plot from titration of heated fraction BM_1 with free xanthine oxidase*

The initial membrane concentration was 24.3 μg of membrane protein/ml which represents 0.045 xanthine oxidase unit/ml originally present before heating. E_f and E_b (defined in the text) were calculated from the increase in NADH oxidase activity as described in the Results section. Each point is the mean of duplicate determinations. The line was fitted by the method of least squares with an ICL 450 computer. Slope = 13.8 ± 1.1, x intercept = −0.115 ± 0.024, correlation coefficient = 0.97 (values ± S.E.M.).

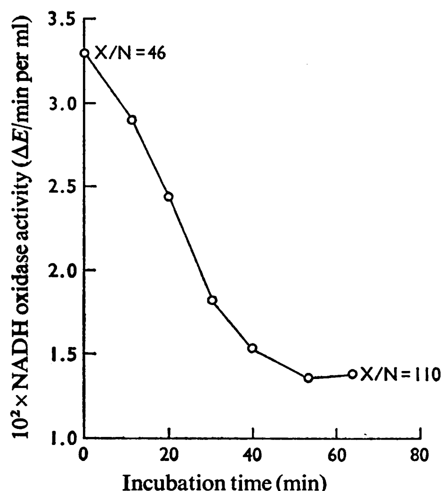

Fig. 7. *Effect of proteolytic digestion on the NADH oxidase activity of fraction* BM_1

A sample (1 ml) of fraction BM_1 was incubated with 1 ml of 0.8% (w/v) pancreatin in 0.05M-sodium pyrophosphate buffer at pH 7.0 containing 1 mM-EDTA at 28°C. Samples were taken at intervals and the NADH oxidase activity was measured. X/N values were calculated from the NADH oxidase activities and the results of a similar experiment in which xanthine oxidase activity was measured.

Proteolytic digestion of fraction BM_1

Incubation of fraction BM_1 with trypsin (or pancreatin), resulted in a progressive decline of NADH oxidase activity to a plateau (Fig. 7). The xanthine oxidase activity of fraction BM_1 was unchanged. During the experiment the X/N value (originally 45) rose to 110. After 80 min soya-bean trypsin inhibitor was added and the digestion mixture was applied to a column of Sepharose 2B. Less than 10% of the xanthine oxidase activity applied was eluted in the void volume, the bulk being eluted later as a single peak (X/N = 89).

Discussion

The association of xanthine oxidase with the milk-fat-globule membrane is well established (Ball, 1939; Morton, 1954; Zittle *et al.*, 1956; Dowben *et al.*, 1967). The present results show that approximately half of the xanthine oxidase present in buttermilk is associated with the particulate fraction BM_1. The chemical composition of this fraction is similar to that of the milk-fat-globule membrane (Table 1). Alkaline phosphatase, which is known to be associated with the fat-globule membrane (Bailie & Morton, 1958) was found associated with fraction BM_1. We therefore consider fraction BM_1 to be representative of the milk-fat-globule membrane.

The remaining xanthine oxidase activity present in buttermilk was found in fraction BM_2 and as free enzyme. Fraction BM_2, which also contains alkaline phosphatase and comprises protein, lipid and phospholipid (but with a protein/lipid ratio greater than that of fraction BM_1), appears to consist of xanthine oxidase with a small amount of associated membrane material. These findings are in general agreement with those of Hayashi *et al.* (1965) who postulated a membrane consisting of a water-insoluble lipoprotein complex surrounding the triglyceride core, and an outer layer of water-soluble lipoproteins.

The ratio of xanthine oxidase activity to NADH oxidase activity (X/N) has been found to be a useful parameter for distinguishing free and membrane-bound xanthine oxidase. The X/N value of the free enzyme is about twice that of membrane-bound xanthine oxidase. Under the standard assay conditions used this difference results from a lower K_m of the membrane-bound enzyme for NADH. The $V_{xanthine}/V_{NADH}$ ratios for fraction BM_1 and purified xanthine oxidase are similar. Also xanthine oxidase activity is unchanged during proteolytic release of the enzyme from the membrane. This shows that the V values for both substrates are unaffected by the association of the enzyme with the fat-globule membrane. Since oxygen is the electron acceptor for both reactions the K_m values of the free and membrane-bound enzyme can be interpreted in terms of the relative affinities of the two forms of the enzyme for each substrate. Thus the lower K_m for NADH and the higher K_m for xanthine of the membrane-bound enzyme represent an enhanced affinity for NADH and a lower affinity for xanthine compared with the free enzyme.

Incremental addition of heated fraction BM_1 to free xanthine oxidase (X/N = 110) results in a progressive decrease in the X/N value to that characteristic of the membrane-bound enzyme (X/N = 50). Enzyme activity (X/N = 45) is associated with material excluded from Sepharose 2B on chromatography of the reconstituted enzyme–membrane mixture, showing that the enhanced NADH oxidase activity is due to the enzyme–membrane association. The recovery of only 20% of the xanthine oxidase activity in the void volume is probably due to dissociation of the enzyme–membrane complex during separation on the column. Thus the enhancement of the NADH oxidase activity is attributable to changes in the affinities of the membrane-bound enzyme and not to the presence of another NADH oxidase activity in the native membrane. The alteration of the X/N value from about 50 to about 110 when free xanthine

oxidase is liberated from the membrane by tryptic digestion confirms this.

The binding of xanthine oxidase to the heated membrane appears to be quite specific. NADH oxidase activity of free xanthine oxidase is not enhanced by the addition of casein, heated fraction BM_2, or by a mixture of protein, triglyceride and phospholipid in the proportions found in fraction BM_1. The enhancement by material excluded from Sephadex G-200 on chromatography of decaseinated skimmed milk (Fig. 3) is probably due to the presence of fat-globules too small to be separated into the cream layer and the membranes of globules ruptured before cream separation.

The enhancement of NADH oxidase activity in the reconstituted enzyme–membrane complex is due to reversible binding of free xanthine oxidase to the fat-globule membrane. There appears to be only one type of binding site and these sites are independent. This is shown by the linearity of the binding plot (Fig. 6). We suggest that xanthine oxidase in the reconstituted enzyme–membrane system binds to the same sites as in the native membrane. Vacant enzyme-binding sites probably exist in the native membrane owing to dissociation of fraction BM_2. The presence of demolybdo forms of the enzyme which are not detectable by assaying xanthine oxidase activity (for a review of various forms of xanthine oxidase see Bray & Swann, 1972) would result in the binding of more than 1 enzyme unit per unit measured in the native membrane. The calculated stoicheiometry was 1.60 xanthine oxidase units per unit present in native fraction BM_1. This is equivalent to 0.114mg of xanthine oxidase bound per mg of membrane protein. The dissociation constant, 0.115 xanthine oxidase unit per ml, is equivalent to 1.61×10^{-8} M (based on a molecular weight of 275000 determined by Andrews *et al.*, 1964).

The NADH activity of xanthine oxidase is well known (Corran *et al.*, 1939; Mackler *et al.*, 1954). Indeed, Avis *et al.* (1956) noted that NADH oxidase activity was higher relative to xanthine oxidase activity in buttermilk than in purified preparations of xanthine oxidase. NADH is considered to bind at a site different from that of other electron-donor substrates (evidence reviewed in Bray & Swann, 1972). The increased affinity for NADH and the decreased affinity for xanthine of the membrane-bound enzyme might thus be attributable to conformational or microenvironmental differences at the catalytic site of the free and membrane-bound enzyme.

Commercially purified xanthine oxidase, prepared by a method involving proteolytic digestion, was used as the source of free enzyme in most of these experiments. Although some workers have reported differences in the properties of xanthine oxidase isolated with and without the aid of proteolytic digestion (Carey *et al.*, 1961; Nelson & Handler, 1968), we found the properties of commercially purified enzyme to be identical with those of the enzyme isolated from skimmed milk without proteolysis. This is consistent with the findings of other workers (Massey *et al.*, 1969; Hart *et al.*, 1970) and indicates that any differences between the two forms are not of major importance in these studies.

The X/N value provides a convenient criterion for distinguishing free and membrane-bound xanthine oxidase in various milk fractions. We have used this criterion to follow the liberation of xanthine oxidase from the membrane and also the reconstitution of the enzyme–membrane complex. Further study of the differences in the catalytic properties of the free and membrane-bound enzyme may lead to a clearer understanding of the nature of the enzyme–membrane association. Also it may prove profitable to use xanthine oxidase as a marker to follow structural rearrangements thought to occur in the plasma membrane when its hydrophilic surfaces appear to directly envelop the hydrophobic triglyceride fat-globule during milk-fat secretion (Henson *et al.*, 1971). Structural changes in the fat-globule membrane caused by various physical treatments of cream and milk (Robert & Polonovski, 1955) such as cooling and agitation could be similarly studied.

We thank Dr. David Gammack of Unigate Ltd. for his suggestions that initiated this work. We also thank Dr. B. B. Gupta of Unigate Ltd. for useful and interesting discussions. The financial support of the Science Research Council and Unigate Ltd. under the C.A.P.S. scheme, and a studentship for M. S. B. is gratefully acknowledged.

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