

PRODUCTION RESEARCH PAPERS

Role of the Isocitrate Dehydrogenases and Other Krebs Cycle Enzymes in Lactating Bovine Mammary Gland

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ABSTRACT

The role of the isocitrate dehydrogenases and other Krebs cycle enzymes in bovine mammary metabolism was studied by investigation of their distribution between cytosol and mitochondria. Citrate synthase was used as a marker for mitochondrial disruption, and distributions were normalized to this enzyme. Aconitase, fumarase, and NAD⁺:malate dehydrogenase were distributed between the mitochondria and the cytosol; evidence for the possible involvement of an aspartate:malate shuttle was also found. The NADP⁺:isocitrate dehydrogenase is predominantly cytosolic with a small but significant amount of mitochondrial component. Using the dye dichlorophenol-indophenol, a low level of NAD⁺:isocitrate dehydrogenase activity was observed in bovine mammary tissues. This assay also allows for detection of the enzyme in fresh mitochondria from a variety of other bovine tissues (heart, liver, kidney, and brain). Activities of the isocitrate dehydrogenases were also examined as a function of gestation and lactation. The NAD⁺:isocitrate dehydrogenase is apparently depressed during gestation with the NADP⁺ form of the enzyme (cytosolic) elevated postpartum. These results indicate that a substantial portion of Krebs cycle activity may become extramitochondrial in bovine mammary gland at the onset of lactation.

INTRODUCTION

Since the original exposition of the citric acid cycle by Krebs and Johnson (20), this cycle has been shown to be an integral part of the energy metabolism of many species. Regulation and control of this pathway can be achieved by constraint of substrates, compartmentation, of the cycle between mitochondria and cytosol (15), or by hormonally induced changes in key enzymes to limit flux through the cycle (27). In the case of the lactating ruminant, where over 65% of an animal's net energy may be diverted to milk synthesis (14), hormonally induced subcellular compartmentation of the Krebs cycle may be important in adaptation to the available substrates. For a recent review of mammary energy metabolism, see Smith et al. (29). The oxidation of D₅-threo-isocitrate to α -ketoglutarate is accomplished in various tissues by three distinct enzymes: an NAD⁺, allosterically regulated enzyme, which is primarily mitochondrial in occurrence (5, 6), and two distinct forms of NADP⁺:isocitrate dehydrogenase, which apparently are selectively localized in either the cytosol or in the mitochondria (25). The subcellular distribution (2, 31) of these and other Krebs cycle enzymes has not been precisely determined for mammary tissue. The objective of this work was to study their subcellular distributions. These data should lead to a better understanding of regulation and control of mammary metabolism in general and of ruminants in particular. In order to assess the particular significance of isocitrate oxidation, it was necessary to adapt a modified assay for NAD⁺:isocitrate dehydrogenase.

MATERIALS AND METHODS

Materials

All coenzymes, substrates, and biochemicals were purchased from Sigma Chemical Company.² All other chemicals were reagent grade.

Samples of fresh bovine mammary tissue and bovine heart were obtained from cows of

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² Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

known good health and productivity through the cooperation of J. E. Keys and A. J. Guidry of US Department of Agriculture, Beltsville, MD. Whole mammary glands were obtained at the time of slaughter, trimmed to remove adipose tissue, and sectioned into pieces approximately $10 \times 10 \times 5$ cm, which were minced and weighed. Subcellular fractionation of mammary tissue was essentially as outlined by Keenan et al. (19). Three volumes (by weight) of cold homogenization medium containing .25 M sucrose, 50 mM Tris pH 7.4, .5 mM EDTA, and 100 μ M dithiothreitol were added and the mixture homogenized for 1 min in a Polytron 10 ST at medium speed. The homogenate was then filtered twice through cheesecloth and cooled on ice. All subsequent operations were carried out at 5°C. The homogenate was centrifuged consecutively at $600 \times g$ for 15 min, $12,000 \times g$ for 30 min, and $40,000 \times g$ for 2.5 h. After each centrifugation, the precipitate was removed, resuspended in homogenization buffer, and washed twice by recentrifugation. Washed pellets were resuspended in homogenization medium at 1/10 of the original volume. The $12,000 \times g$ pellet was examined by electron microscopy and found to represent primarily mitochondria. The $40,000 \times g$ supernatant showed very little change in the specific activity of the Krebs cycle enzymes in the $12,000 \times g$ supernatant. Thus, the $12,000 \times g$ supernatant values are taken to represent cytosol.

Due to the distance between the place of slaughter and our laboratory, tissues from lactating cows were processed on site, subcellular fractions obtained, and samples then frozen for 1 d prior to assay. Fresh bovine heart, kidney, liver, and brain from meat animals were obtained from a local slaughter house and placed directly on ice for transport to the laboratory (15 min). Rat mammary tissue was excised in our laboratory and treated as was bovine mammary tissue, but enzyme activities were assayed immediately.

Experiments on activity as a function of stage of gestation and lactation were on frozen crude precipitates ($10,000 \times g$ for 30 min) and supernatants obtained from John Keys. In this case, the precipitates were first diluted 1 to 4 in the homogenization buffer, homogenized gently by hand, and then briefly sonicated

before assay.

Enzyme Assays

Succinate dehydrogenase was assayed by the method of Pennington (23) using a zero time blank and a 15-min incubation. All other enzyme assays were at 25°C in 1-cm cells and monitored on a Gilford spectrophotometer with a scale setting of 0 to .200 absorbance units. Time courses of the reaction were linear for the period of the assay. One enzyme unit catalyzes the formation of 1.0 μ mol of product/min at 25°C. Specific activity is defined as enzyme mU/mg protein. Krebs cycle and related enzymes were assayed in their respective incubation mixtures as follows: citrate synthase (28), aconitase (10), NADP⁺:isocitrate dehydrogenase (11), fumarase (17), NAD⁺:malate dehydrogenase (9), aspartate amino transferase (1), glucose 6-phosphate dehydrogenase (22), glutamic dehydrogenase (32), and NADP⁺:malic dehydrogenase (18).

Activity of NAD⁺:isocitrate dehydrogenase was assayed by the method of Cook and Sanwal (7), using excess "diaphorase" and coupling the oxidation to the dye dichlorophenol-indophenol (DCPIP). Conditions used for assay (substrate, ADP, metal ion concentration, and buffer) were those of Plaut (24). Decrease in absorbance at 600 nm was measured as described.

Protein Determination

Protein concentrations were determined by the method of Bradford (4) with appropriate buffer blanks and with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Assay for Nicotinamide Adenine Dinucleotide⁺:Isocitrate Dehydrogenase

The discovery that NAD⁺:isocitrate dehydrogenase (IDH) is allosterically controlled by ADP and ATP (5) has led to the belief that this enzyme plays an important regulatory role in Krebs cycle metabolism. In our initial studies on bovine mammary gland we found virtually no NAD⁺:IDH activity when employing the standard spectrophotometric assay at 340 nm.

Fresh steer heart mitochondria, a relatively rich source of NAD⁺:IDH, were tested using the absorbance (A₃₄₀) assay, and the results were also negative; neither freeze thawing nor sonication yielded positive results. The ratios of NAD⁺:NADP⁺ isocitrate dehydrogenases in animals have for the most part been obtained with solubilized extracts of acetone powders of mitochondria or of total homogenates (30). A highly active NADH:oxidase was found in heart. When isocitrate oxidation through NAD⁺ was measured in the presence of both diaphorase and DCPIP, significant ADP-dependent NAD⁺:IDH activity was found in fresh bovine heart mitochondria (Table 1). Omission of the diaphorase is nearly compensated for by the endogenous NADH oxidase activity. Similar studies were conducted on fresh mitochondria from steer liver and kidney. These tissues were also negative for assays conducted at A₃₄₀, but they gave reasonable activity when DCPIP was employed. In the case of brain, which in rat has been reported to be high in NAD⁺:IDH, the A₃₄₀ assay gave positive results. However, the DCPIP assay detected nearly five times the activity. It appears as though a dye-coupled assay may be necessary to quantify the NAD⁺:IDH activity present in a variety of tissues. Miscalculation of this important regulatory enzyme could interfere with metabolic simulations. The activity of the heart enzyme remains relatively constant after storage for 1 d and drops about one half after 3 mo at -20°C.

Relative Ratios of Isocitrate Dehydrogenases in Fully Lactating Animals

Having established the conditions for assay of the IDH activities in bovine tissue, heart and mammary gland mitochondria from the same fully lactating animal were prepared, frozen, transported to the laboratory, and then assayed the following day for dehydrogenase activities after thawing and sonication. Succinate dehydrogenase is shown as a marker for mitochondria (Table 2). Bovine heart mitochondria have a ratio of about 25:1 for NADP⁺/NAD⁺:IDH activity as measured on samples that were frozen overnight and thawed and sonicated prior to assay. Freshly prepared and sonicated mitochondria from bovine tissues exhibit little or no loss of the NAD⁺:IDH activity on storage for 1 d (Table 1), while the NADP⁺:IDH activity actually increases about 10% under these conditions. The NADP⁺/NAD⁺:IDH concentrations in mammary mitochondria would be roughly 12:1. Thus, mammary and heart mitochondria prepared from the same lactating cow have a very dissimilar NADP⁺/NAD⁺:IDH ratio, and overall, total heart mitochondrial IDH activity is 13 times greater than the total mammary mitochondrial IDH concentration. Experiments in which mixtures of mammary and heart preparations were coincubated showed no inhibition of NAD⁺:IDH activity, indicating that the lower NAD⁺:IDH found in the mammary gland is not the result of endogenous inhibitors.

TABLE 1. Activity of nicotinamide adenine dinucleotide⁺:isocitrate dehydrogenase in fresh and frozen mitochondria from bovine tissues.

Conditions	Specific activity				
	Heart ¹	Kidney ²	Liver ²	Brain ²	
	(mU/mg protein)				
	\bar{X}	SD			
Complete assay first day	23.3	3.3	5.64	4.06	45.1
Complete assay 24 h frozen and sonicated	23.5	3.2	5.14	3.98	59.7
No ADP, 2nd d	12.8	1.6	2.81	3.47	50.5
No isocitrate, 2nd d	.3	.3	.01	.01	.3
No diaphorase, 2nd d	19.9	4.3	5.47	2.72	57.3
Frozen and sonicated, 3 mo	10.7	2.6	...		

¹ Average of three preparations from three animals.

² One preparation each, assayed in triplicate.

TABLE 2. Distribution of dehydrogenase activities among frozen fractions from bovine tissues.¹

Distribution	Specific activity		
	Succinate dehydrogenase	NADP ⁺ :IDH ²	NAD ⁺ :IDH
	(mU/mg protein)		
Heart			
600 × g Supernatant	30.0	135	9.27
12,000 × g Pellet	140	414	16.5
12,000 × g Supernatant	4.0	114	3.30
Mammary			
600 × g Supernatant	3.0	607	.70
12,000 × g Pellet	35.1	30	2.53
12,000 × g Supernatant	7.0	590	1.03
Heart and mammary			
12,000 × g Pellets	16.3

¹ Both heart and mammary tissues were from the same lactating cow; the fractions were frozen and assayed the following day.

² IDH = Isocitrate dehydrogenase.

Mitochondria were also prepared from lactating rat mammary gland. No activity for NAD⁺:IDH was observed with the A₃₄₀ assay; using the DCPIP assay, significant NAD⁺:IDH activity was observed in rat mammary mitochondria (Table 3). However, the cytosolic NADP⁺:IDH is much lower in rat than in bovine mammary gland.

Changes in the Activities During Mammary Development

Development of the mammary gland, particularly in primiparous animals, requires a number of metabolic and structural changes. In the fully lactating animal, the mitochondrial forms of IDH (both NAD⁺ and NADP⁺) predominate in the heart, while the cytoplasmic form predominates in the mammary gland (Table 2). Studies of changes in the IDH as a function of time of gestation and lactation might shed some light on the relative expression of these enzymes. Assay of the postmitochondrial supernatants and precipitates from mammary gland showed a dramatic increase of soluble NADP⁺:IDH at the onset of lactation (Figure 1A). The mitochondrial activity for succinate dehydrogenase also increased at lactation, but not as dramatically (Figure 1B). The NAD⁺:IDH initially occurred at a low concentration, then decreased during gestation.

In contrast, the NADP⁺ activity in the crude mitochondria (precipitate) remained relatively constant throughout (Figure 1A). It would appear that in ruminant mammary tissue the changes observed in the activities of these enzymes are related to hormonal changes accompanying gestation and parturition, possibly resulting in a switch to an extramitochondrial flux of metabolites for a portion of the Krebs cycle. Because heart tissue from the same fully lactating animal showed significant NAD⁺:IDH activity, it would seem that there may be repression of this enzyme in the de-

TABLE 3. Distribution of isocitrate dehydrogenase activities from lactating rat mammary gland.

Distribution	Specific activity ¹	
	NAD ⁺ :IDH ²	NADP ⁺ :IDH
	(mU/mg protein)	
12,000 × g Pellet	11.6	39.7
12,000 × g Supernatant	.10	77.4

¹ Averages from two animals 14 d lactating.

² IDH = Isocitrate dehydrogenase.

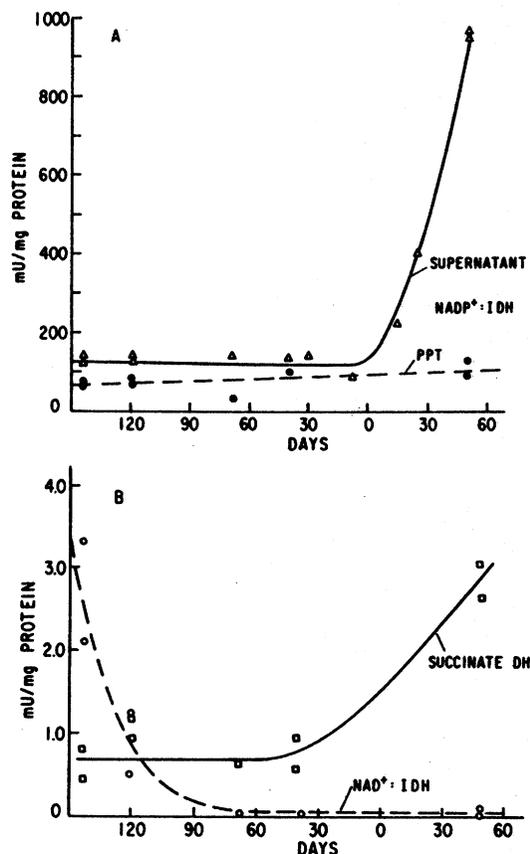


Figure 1. Changes in the activities of selected dehydrogenases as a function of stage of gestation and lactation. Assays were conducted as described on A) nicotinamide adenine dinucleotide⁺: isocitrate dehydrogenase (NADP⁺:IDH) for both postmitochondrial supernatants (Δ) and mitochondrial pellets (●); and B) mitochondrial pellets for NAD⁺:IDH (○) and succinate dehydrogenase (□). Data are plotted in days prepartum and postpartum.

veloping mammary gland. In contrast, NAD⁺:IDH is significantly expressed in the lactating rat mammary gland (Table 3).

Subcellular Distribution of Krebs Cycle Enzymes in Lactating Bovine Mammary Gland

To understand better the roles of the isocitrate dehydrogenases in ruminant mammary metabolism, their total activities and intracellular compartmentation were compared with other Krebs cycle enzymes. The total activities of Krebs cycle and related enzymes in homogenates of lactating bovine mammary gland are

in Table 4. For comparison with previous partial compilations (2, 8, 26, 30), data are expressed as micromoles per minute per gram of tissue and adjusted to 37°C. The values for aconitase, fumarase, and NAD⁺:IDH are reported here for the first time. Other activities found in this study are in good agreement with those in the literature, except for malate dehydrogenase, which appears quite variable. The lowest activities appear to be (with the exception of citrate synthase) associated with enzymes, which are thought to occur exclusively within mitochondria (15). Therefore, a survey of the distribution of Krebs cycle enzymes between cytosol and mitochondria was undertaken. Tissue was obtained from three cows that were in midlactation and free of infection. The data for mitochondria and cytosol are in Table 5. Specific activities of the intramitochondrial portion of the Krebs cycle show that in terms of overall synthetic capabilities, the total IDH (NAD⁺ + NADP⁺) is lowest and may represent the limiting enzyme(s) within the mitochondrial pathway. As shown, the NAD⁺ may be repressed and the mitochondrial NADP⁺ may not be stimulated during mammary development.

When citrate synthase was used as a marker for mitochondrial disruption, as suggested by Fatania and Dalziel (12), succinate dehydrogenase increased to 130%, indicating about 30% breakage of mitochondria. Taking this into account, about 65% of the aconitase and 95% of the NADP⁺:IDH activity is cytosolic in nature. The NAD⁺:IDH was low and the values were erratic even employing the DCPIP assay. Table 2 shows the highest value found, but the NAD⁺:IDH activities for all lactating cows studied ranged from .1 to 2.5. Whatever the actual number, when combined with NADP⁺:IDH, the mitochondrial potential of this step in the cycle is small when compared with the cytosolic NADP⁺:IDH activity and the specific activities of other mitochondrial enzymes. Thus, diversion of a high percentage of metabolic flux from the mitochondria could occur in the lactating bovine mammary gland. As seen previously, this increase would be coincident with the onset of full lactation. In computer models for mammary metabolism, only NADP⁺:IDH has been considered extramitochondrial (31). The extramitochondrial occurrence of aconitase and fumarase had not been reported

TABLE 4. Activities of Krebs cycle and related enzymes in homogenates of lactating bovine mammary gland.

Enzyme	Reference				This study ¹	
	(8, 26)	(31)	(2)			
	(μmol/min per g tissue at 37° C)				\bar{X}	SE
Citrate synthase	13.2		3.3	7.37	1.03	
Aconitase				2.45	1.18	
NADP ⁺ :IDH ²	24.5	58.8	59.9	43.2	15.7	
NAD ⁺ :IDH				.14	.10	
α-Ketoglutarate dehydrogenase	.22		.35			
Succinate dehydrogenase		.07		.56	.28	
Fumarase				7.42	2.18	
Malate dehydrogenase	110	9.66	41.6	23.8	8.42	
Aspartate aminotransferase		4.56	6.33	7.80	3.86	
Glutamic dehydrogenase	3.6			.10	.05	

¹ Averages from three different cows at midlactation all in good health with moderate milk yields.

² IDH = Isocitrate dehydrogenase.

previously. The cytosolic NADP⁺:IDH (3) has been postulated to be a potential source of reducing equivalents necessary for *de novo* synthesis of fatty acids and cholesterol in bovine mammary gland. The ratio of cytosolic NADP⁺:IDH to glucose 6-phosphate dehydrogenase was found to be 33:1, supporting this latter hypothesis, at least from the point of metabolic potential. This value is close to that of 26:1 previously given (31).

There has also been speculation that mitochondrial NADP⁺:IDH could generate citrate by reversing the Krebs cycle. At first glance, the enzyme's low synthetic capability, coupled with relatively high total isocitrate concentration (2), would seem to argue against this pathway. Hardwick (16), however, presented clear evidence that perfused goat mammary gland could incorporate ¹⁴CO₂ into citrate via α-ketoglutarate. The cycle could be reversed

TABLE 5. Krebs cycle enzymes in mitochondria and cytosolic fractions from lactating bovine mammary gland.

Enzyme	Mitochondria				Cytosol		
	Specific activity ¹		Ratio ²	% Particulate ³	Specific activity ¹		% Soluble ³
	(nmol/min per mg protein)				(nmol/min per mg protein)		
	\bar{X}	SE			\bar{X}	SE	
Citrate synthase	790	230	6.7	100	30.7	25.4	7
Aconitase	53.9	8.8	1.6	24	31.8	10.9	65
NADP ⁺ :IDH	28.8	9.0	.1	2	715	109	95
NAD ⁺ :IDH	1.4	.9	3.7	80	.6	.6	0
Succinate dehydrogenase	89.4	13.3	10.1	130	2.6	1.2	0
Fumarase	489	92	4.5	68	89.0	14.3	30
NAD ⁺ :malate dehydrogenase	1550	170	5.9	66	248	32	33

¹ Averages from three different cows at midlactation all in good health with moderate milk yields.

² Ratio of specific activity in mitochondria to that found in the total homogenate.

³ Percentage of total activity present normalized to citrate synthase.

TABLE 6. Distribution of activities of enzymes of malate aspartate shuttle in fractions of lactating bovine mammary gland.

Distribution	Specific activity					
	Succinate dehydrogenase		NAD ⁺ :malate dehydrogenase		Aspartate aminotransferase	
	(mU/mg protein)					
	\bar{X}	SE	\bar{X}	SE	\bar{X}	SE
600 X g Supernatant	7.0	.9	263	47	76.4	7.3
12,000 X g Pellet	89.4	13.3	1550	170	444	100
12,000 X g Supernatant	3.5	.5	221	10	54.8	9.8
% Soluble ²	0%		33%		35%	

¹ Samples as described in Table 5.

² Normalized as described in Table 5.

within the mitochondria by NADP⁺:IDH; lack of the irreversible NAD⁺:IDH prevents competition for the newly formed isocitrate, and so reversal here could contribute to the net export of citrate into milk (13). In addition, the absence of the citrate cleavage enzyme (which requires ATP) is well-documented for bovine mammary gland (2, 3). Clearly, the apparently low NAD⁺:IDH argues against significant contribution to NADH production by a proposed NADPH-NADH shuttle (16) employing only isocitrate dehydrogenases.

The relatively high specific activity of NAD⁺:malate dehydrogenase and its distribution between mitochondrial and cytosolic fractions suggests that a malate-aspartate shuttle might be a part of mammary mitochondrial metabolism so that NADH produced in the cytoplasm could be shuttled into the mitochondria. For this to occur (21), both malate dehydrogenase and aspartate aminotransferase must be present in both mitochondrial and cytosolic compartments. Table 6 shows that this is the case and points toward further extramitochondrial Krebs cycle activity in lactating bovine mammary gland. Further experiments on intact mitochondria are necessary to substantiate this hypothesis.

In summary, in fresh or frozen preparations of bovine tissues, also at least in rat mammary gland, estimation of NADH producing enzymes must be done using a dye coupled assay when comparable NADH oxidase activity is present. In the case of NAD⁺:IDH, this results in a more reasonable estimation. In contrast,

NAD⁺:malate dehydrogenase is present in bovine mammary gland at such high concentrations that effects of NADH oxidases are minimized. The data confirm the speculation that the cytosolic form of NADP⁺:IDH predominates in ruminant mammary gland. In rat mammary gland, however, both NAD⁺ and NADP⁺:IDH occur in the mitochondria and are significant parts of the Krebs cycle. The cytosolic NADP⁺:IDH is under hormonal control and in bovine mammary gland increases with increasing gestation and lactation, as does succinate dehydrogenase. In contrast, low NAD⁺:IDH is apparently further depressed at lactation. Comparison of the distribution of the Krebs cycle enzymes in lactating mammary gland indicates that a relatively high percentage of flux through the cycle may occur outside of the mitochondria. A high citrate synthase activity coupled with reversible NADP⁺:IDH allows for the known net export of citrate; a high degree of conversion of citrate to α -ketoglutarate may thus occur outside of the mitochondria to potentially produce NADPH for *de novo* fat synthesis. On the other end of the cycle, high levels of NAD⁺:malate and aspartate aminotransferase indicate a possible involvement of the malate and aspartate shuttle in bovine mammary metabolism.

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