CHANGES IN ASCORBIC ACID CONTENT AND ACETYLCHOLINESTERASE ACTIVITY IN THE MUSCLE OF FROG FOLLOWING SCIATECTOMY

T. SOMASEKHAR AND R. V. KRISHNAMOORTHY

Department of Zoology,
University of Agricultural Sciences,
G.K.V.K. Campus, Bangalore - 560 065

(Received on November 10, 1983)

Summary: 1. Unilateral sciatectomy for three months in the frog, Rana cyanophlyctis resulted in a substantial increase on unit weight basis in the ascorbic acid (ASA) and dehydroascorbic acid (DHA) contents of the sciatectomized gastrocnemius muscle. Diketogulonic acid (DKA) levels did not vary. On whole muscle-weight basis only the ASA level increased.

2. The AChE activity in sciatectomized muscle is significantly lower than that of the control.

3. Partially purified preparation of the AChE from the sciatectomized muscle showed different kinetics compared to that from innervated control.

4. In vitro additions of ASA in physiological concentration to the enzyme assay medium inhibited the AChE activity significantly and the inhibition was an un-competitive type.

5. Reduced activity of the enzyme has been correlated to the increased concentration of ASA in the sciatectomized muscle.

Key words: ascorbic acid, frog gastrocnemius muscle, dehydroascorbic acid, AChE activity, sciatectomy, loss of trophic influence

INTRODUCTION

Recent studies revealed that the release of transmitters in nerve terminals is under the control of ascorbic acid (1, 3, 14). However, it is not clear whether the ascorbic acid has any influence over the AChE activity in the muscle. The ascorbic acid accumulates in the muscle during denervation (8) and the AChE activity changes on time course of denervation (Somasekhar, unpublished data). The acetylcholine content decreases in the muscle after denervation (9). In order to study more precisely, the effects of ascorbic acid and its metabolites on the AChE activity of the muscle and their role in the muscular denervation-atrophy, it is desirable to investigate the in vitro effects of ascorbic acid on the AChE activity of the muscles. This paper describes some of the work done in order to achieve these goals.
MATERIAL AND METHOD

Male frogs, *Rana cyanophlichtis* in a range of 20–25 g body weight were used for experiments. The unilateral sciactectomy (alternatively right or left) was done as described earlier (10) three months before the gastrocnemius muscles were removed under light anaesthesia with ether. The frogs were maintained in large aquarium tanks, allowing free movement and force-fed every alternate day with sliced earthworms *ad libitum*.

The excised gastrocnemii were weighed immediately and then chilled on ice-cubes before they were used for further experiments. The endogenous levels of Ascorbic acid (ASA), Dehydroascorbic acid (DHA) and Diketogulonic acid (DKA) in the muscles were estimated colorimetrically according to Roe et al. (16).

The AChE of muscles was partially purified through ammonium sulphate fractionation as described by Habibulla and Newburg (5). The partially purified enzyme was dialyzed against glass distilled water. Dialysis was carried out in cellophane tubes (0.5 cm diameter, 15 cm long) at 4°C for 12 h suspended in 2 L distilled water. The dialysing fluid was changed every 3 h. The AChE was assayed colorimetrically according to Hestrin (6). The protein in the enzyme extract was estimated colorimetrically (13) using bovine serum albumin as a standard.

The AChE activity in extraneously added ASA-medium was assayed in order to study the effects of ASA on the kinetics of the enzyme. 300 μg L-ascorbic acid (product of Sigma Co.) was added to one ml of the assay medium for these kinetic studies. This concentration was chosen as the endogenous level of total ASA in the muscle, as the total ASA level in the muscles of laboratory frogs vary between 160–300 μg/muscle (8). The Michaelis-Menten kinetics of the AChE were studied both in the presence of ASA as well as in the medium without ASA.

RESULTS

The scheme adopted for purifying the muscle AChE partially in the present study yielded 53.39% protein and 6.35 times greater enzymic activity. The fold purification was found to be about 6.35 and consistent for both the muscle preparations. The dialysis of the ammonium sulfate fractionated protein yielded nearly 1.9-fold higher enzyme activity and about 75.45% protein.

ASA in cells will be oxidized reversibly and enzymatically to unstable DHA; the letter on hydrolysis irreversibly yields DKA (2). In active tissues these three metabolites occur in varying proportions, depending on the extent of oxidations and hydrolysis to
TABLE I: Protein yield and AChE activity in the purification steps of the method adopted (see text for details) in a representative assay.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Step</th>
<th>Volume of fraction in ml</th>
<th>Protein concentration mg/ml</th>
<th>Total amount mg</th>
<th>Enzyme specific activity</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Crude homogenate</td>
<td>200</td>
<td>0.457</td>
<td>91.4</td>
<td>10.9</td>
<td>1.0</td>
</tr>
<tr>
<td>2.</td>
<td>Ammonium sulphate fraction</td>
<td>100</td>
<td>0.324</td>
<td>64.8</td>
<td>38.8</td>
<td>3.56</td>
</tr>
<tr>
<td>3.</td>
<td>Ammonium sulphate fraction after dialysis and centrifugation</td>
<td>50</td>
<td>0.244</td>
<td>48.8</td>
<td>69.3</td>
<td>6.35</td>
</tr>
</tbody>
</table>

TABLE II: Endogenous levels of Ascorbic acid metabolites in the gastrocnemius muscle of frogs sciactomized for 3 months.

<table>
<thead>
<tr>
<th>Muscles</th>
<th>ASA μg/g muscle ± S.D. n=6</th>
<th>DHA μg/g muscle ± S.D. n=6</th>
<th>DKA μg/g muscle ± S.D. n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Innervated</td>
<td>67.4±10.9 (11.79±1.9)</td>
<td>89.1±25.3 (15.59±4.42)</td>
<td>89.5±28.1 (15.66±4.91)</td>
</tr>
<tr>
<td>Sciactomized</td>
<td>130.6±17.8 (16.95±2.30)</td>
<td>147.1±28.2 (19.12±3.66)</td>
<td>97.5±23.3 (12.67±3.01)</td>
</tr>
<tr>
<td>Incidence of change on sciactomy* (%) change over control</td>
<td>Increase</td>
<td>Increase</td>
<td>No change</td>
</tr>
<tr>
<td>P&lt;0.001</td>
<td>P&lt;0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parenthesis are the levels per whole muscle (X ± S.D., n=6).

*When the concentrations per unit wet weight were compared.

which the cells are subjected. All these three metabolites occur in the gastrocnemius muscle of frog. In the innervated muscle the DHA and DKA concentrations were nearly equal and greater than that of ASA (Table II). Sciactomzy increased the levels of ASA and DHA in the muscle per unit weight basis but not the DKA level. Sciactomized muscles for 3 months showed 26±2% atrophy (loss of muscle mass) and when expressed on whole muscle basis the ASA level significantly increased (P<0.001), but the DHA and DKA levels did not vary much.

The ASA/DHA ratio in the innervated muscle is 0.753 and in sciactomized muscle, 0.884. Greater ratios indicate more of ASA concentration existing in the muscle relative...
to that of DHA. Sciactectomized muscle showed greater ratios indicating probably poor oxidation of ASA in the muscle. DHA/DKA ratio in the innervated muscle is 0.994 and in sciactectomized muscle. 1.509. Higher ratios indicate, more DHA concentration relative to that of DKA; hence poor hydrolysis of DHA into DKA would have resulted in the muscle.

Table III presents the data on *in vitro* additions of ASA on the AChE activity. It is clear from this table that the sciactectomy decreases the specific activity of the enzyme. Sciactectomy brought forth about 16% reduction in the enzyme activity. In the presence of ASA, this reduction was more (i.e., about 18%) due to sciactectomy. *In vitro* addition of ASA inhibited the AChE activity considerably irrespective of the nature of the muscle. There was about 20% inhibition of activity in the innervated muscle enzyme and about 24% in the sciactectomized muscle enzyme.

When ASA concentration was varied (from 0 to 300 µg/ml) in the assay medium, the degree of inhibition did not vary much (P > 0.01). As ASA is known to be a powerful reducing agent (2), it is felt that the inhibition caused here, could be due to the reducing nature of the ASA. But when tested it was found that AChE specific activity was not altered by the additions of other reducing agents like KCN, NADH, NADPH, strontium chloride and mercaptoethanol.

![Graph](image)

*Fig. 1: Substrate versus rate of enzyme activity profiles for AChE purified from gastrocnemius muscle of frog sciactectomised for 3 months. 300 µg of Ascorbic acid (ASA) was added to whole assay medium to study the influence of ASA on the enzyme activity. The enzyme was assayed at 4 mM Ach, 26°C and pH 7.1.*
Figure 1 demonstrates the effect of ACh (substrate) concentration on the muscle AChE activity. Both the enzyme preparations i.e., from innervated as well as from sciaticotomized muscles showed a significant inhibition, when the physiological concentration of ASA (300 μg) was added to the whole assay medium. Sciactectomy brought forth more ASA influence on the enzyme activity. Fig. 1 also illustrates that the sciactotomized muscle-enzyme is substrate inhibited and has a lower substrate optimum than those of the innervated control muscle.

![Graph showing the effect of ACh concentration on AChE activity with and without ASA.](image_url)

**Fig. 2:** 1/v Vs 1/(S)^4 plots of the data presented in Fig. 1.

**TABLE III:** Influence of *in vitro* additions of Ascorbic acid on the gastrocnemius AChE* activity of frogs, sciactotomized for 3 months.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>AChE activity: nano mole ACh hydrolysed mg protein/minute ((\bar{x} \pm S.D., n=6))</th>
<th>Incidence of change on ASA <em>in vitro</em> additions.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without ASA</td>
<td>With ASA (300 μg/ml)</td>
</tr>
<tr>
<td>Innervated</td>
<td>46.0 ± 2</td>
<td>35.6 ± 1.7</td>
</tr>
<tr>
<td>Sciactotomized</td>
<td>38.8 ± 1.7</td>
<td>29.2 ± 1.6</td>
</tr>
<tr>
<td>Incidence of change on sciactectomy</td>
<td>Decrease</td>
<td>Decrease</td>
</tr>
</tbody>
</table>

*assayed at 4 mM ACh, 26°C and pH 7.1.
An attempt to establish Lineweaver-Burk plots and study the kinetics of enzyme for both the muscle enzymes has become futile, because the enzyme is not obeying the Michaelis-Menten rules. The double reciprocal plots were not at all getting linearized. The data in Fig. 1 point out to a clear sigmoid response rather than a linear or regular hyperbolic response of S versus V profile. Therefore the sigmoid kinetics were applied to the data by establishing 1/V versus 1/[S]^4 plots (Fig. 2) and also through Hill equation (17) (Table III). 1/V versus 1/[S]^4 plot was the best linear fit obtained (Fig. 2) to arrive at $V_{\text{max}}$ and apparent $K_m$. As is clear from the linear plots of 1/V versus 1/[S]^4, there are changes in $V_{\text{max}}$ and apparent $K_m$ of muscle AChE activity with reference to sciatetomy as well as ASA-influence (see Table IV). Sciatetomy lowered the $V_{\text{max}}$ and increased the $K_m$ for AChE. Using Hill equation (17) the $K_m$ values ($K'$ in Table IV) were calculated and as stated by Segal (17), and as expected they are slightly higher than the apparent $K_m$ derived from the 1/V versus 1/[S]^4 plot (Table IV).

TABLE IV: Kinetic parameters of the AChE purified from the control and sciatetomized frog muscles.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{\text{max}}$</th>
<th>$K'$ calculated from Hill's equation</th>
<th>$K'$ (graphical) (see fig. 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Innervated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without ASA</td>
<td>51.28</td>
<td>2.13</td>
<td>1.8</td>
</tr>
<tr>
<td>With ASA</td>
<td>42.55</td>
<td>2.23</td>
<td>1.7</td>
</tr>
<tr>
<td>Sciatetomized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without ASA</td>
<td>47.62</td>
<td>2.17</td>
<td>2.0</td>
</tr>
<tr>
<td>With ASA</td>
<td>42.55</td>
<td>2.32</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*read from 1/[S]^4 vs 1/V plot and bear the units: nM ACh hydrolysed/min^-1 1/mg protein.

$K'$ is the apparent $K_m$ in mm ACh.

In general, the ASA addition to the assay medium reduced the $V_{\text{max}}$ and increased the $K_m$ in both the muscle preparations (Table IV). As high $K_m$ represent the low affinity of the enzyme to the substrate, the ASA seems to lower the affinity of the enzyme. The 1/V versus (1/[S]^4 plots for both the muscles, show varying kinetic parameters with reference to ASA-inhibition (Fig. 2). Hence the type of inhibition of AChE on control and sciatetomized muscle, in the presence of ASA may be an un-competitive one (17).

DISCUSSION

Dialysis of the partially purified enzyme preparation seems to accelerate the enzymic activity (Table I). Obviously, this illustrates the occurrence of possible dializable inhibitors
in the enzyme obtained after ammonium sulphate fractionation. When examined, we noticed that the ASA is dializable and the dialized enzyme preparation is free from ASA contamination. Studies on such ASA-free preparation, have ruled out the interferences by enzyme activations either due to endogenous levels of the ASA or of its cumulative levels on account of extraneous additions.

The data on ASA/DHA and DHA/DKA ratios suggest the catabolic patterns of the ASA in the sciatactomized muscles. Poor oxidation of ASA in the sciatactomized muscle may have several implications. Poor oxidation of ASA would increase the endogenous level of ASA in the muscle. As ASA is not synthesized in the muscle (12), all the ASA content of the muscle must have its origin from tissue stores like liver, adrenals etc. Krishnamoorthy and Satyam (8) found no changes in the catabolic patterns of ASA in the denervated muscle. The observed differences in the muscle following sciatactomy, therefore show that the latter alters the permeability of the muscle membrane.

Earlier studies in this laboratory substantiated that long-term sciatactomy in frogs results in the atrophy of gastrocnemius muscle (11) and the atrophied muscle is characterized by a significant decrease in ACh content and lowered AChE activity as a function of time of sciatactomy (Somasekhar, unpublished data). The results presented in the present study indicated that the AChE of sciatactomized muscle differs in enzyme kinetics; particularly with reference to un-competitive inhibition by ASA. The cause for these changes must be sought in the configurational changes following sciatactomy occurring in AChE molecules. There is evidence now that AChE exists in multimolecular forms which are in turn regulated by innervation patterns (19). Loss of trophic influence (4) effected through sciatactomy might have been responsible for these changes in the kinetics of AChE.

Another feature found in the results is the importance of ASA in the maintenance of ACh activity in the muscle. It is clearly shown that ASA inhibits AChE activity and alter its kinetics. This is particularly interesting to note as ASA is known to promote the activities of many enzyme systems like the phosphatases (12), alkaline phosphatase (7) and inhibit hepatic catalase (18). However, how these inhibition and promotion of enzyme activities by the ASA are effected are not clear.

Chen-Hui and Yoshida (1) reported that ASA is an endogenous factor in releasing ACh from synaptic vesicles. It is thus interesting to note that while the endogenous ASA releases the ACh from synaptic vesicles (op. cit.), it inhibits the AChE activity of the muscle (present results).

How the ASA accumulates in the atrophied muscle is not clear. Krishnamoorthy and Satyam (8) suggested that permeability changes occurring at muscle membrane following denervation permit the blood ASA to accumulate inside the sarcoplasm. While this is the only suggestion found to explain the occurrence of higher concentration of
ASA in the atrophic muscle, the increased endogenous synthesis of ASA in the frog muscle tissue following denervation cannot be totally ruled out. It is not known whether the frog can synthesize ASA in its tissue like any other vertebrate or is dependent on dietary resources like rat and primates (15). But presence of ASA metabolites like DHA and DKA in the muscles shows the existence of active ASA metabolism in frog muscles which are at the fulcrum of trophic influences of the nerve, innervating the gastrocnemius muscle.

ACKNOWLEDGEMENTS

We thank the CSIR for the award of a Senior Research Fellowship to one of us (TS). Our thanks are also due to Dr. R. Narayana, DI BSH, University of Agricultural Sciences, Bangalore for facilities and encouragement.

REFERENCES