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Autocatalytic pathways to cell death: a new analysis of the tuna burn problem

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Abstract

During capture and storage of tuna, a small but significant number of fish display a characteristic muscle degeneration termed tuna burn. Based on detailed amino acid analyses and on previous studies of metabolite changes during online swimming of tuna, a new model of the etiology of burnt muscle is developed. According to this model oxygen-lack to white muscle (developing initially during capture) leads to a metabolic collapse, to a drop in ATP concentration, to a consequent opening of ATP-dependent K^+ channels, with an efflux of K^+, and thus to a collapse of membrane potential. When the membrane potential falls far enough to open voltage-dependent Ca^{++} channels, Ca^{++} influx occurs leading to elevated Ca^{++} concentrations in the cytosol. This process is augmented by simultaneous movement of Ca^{++} from sarcoplasmic reticulum (SR) and from mitochondria into the cytosol. At high intracellular concentrations Ca^{++} can be devastating. One of its more notable effects involves the activation of Ca^{++}-dependent proteases, which preferentially target key components of the contractile machinery (troponins, tropomyosin, C-protein, M-protein, Z-discs, α-actinin) and thus cause disassembly of myofilaments prior to any significant hydrolysis of myosin or actin. This process is autocatalytic in the sense that Ca^{++}-activated proteases may act upon SR, thus increasing Na^+/Ca^{++} exchange, and ultimately adding more Ca^{++} to the cytosolic pool. According to this model, the difference between burnt and unburnt regions of the myotome is simply due to how far each region has moved along this self-destructive, autocatalytic pathway. The model is helpful in explaining previously perplexing data and in making useful (i.e. measurable) predictions for further studies of this important problem.

Introduction

Tuna burn is a term that has been coined for a state of muscle deterioration which is characterized by the development of a color that is paler than usual and a soft texture that implies some self-digestion. In Japanese literature, the analogous term is "yake", which literally means "spontaneously done meat" (Konagaya and Konagaya 1979). Although the time course of tissue deterioration is not precisely known, it appears to take at least several hours after capture to develop, and usually even longer. The extent of damage varies greatly, from as little as about 5% of the expaxial muscle mass to essentially 100% of it (Cramer *et al.* 1981). In addition, burnt tuna muscle is known to have high con-

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centrations of lactate and the pH of extracellular fluid (ECF) is about pH 5.5–6 (Cramer *et al.* 1981). The latter is usually considered to reflect acidic intracellular fluid (ICF) which in turn is assumed to favor lysosomal acid proteases. In support of this assumption, direct measurements of soluble protein (as water extractable protein nitrogen) indicate that severely damaged samples sustain reductions down to about 50% of control values (Nakamura *et al.* 1977). The implicit model of tuna burn, then, favored by most earlier workers in this field can be summarized in the following metabolic scheme:

metabolic acidosis → activation of acid proteases → self-digestion → muscle deterioration (burn)

To properly appreciate this proposed etiology of tuna burn, it is important to recall the metabolic conditions of the organism when captured online and brought on board a vessel. Because these teleosts are vigorous swimmers, perhaps unsurpassed in speed and manoeuverability, it is not surprising that after even a few minutes of online swimming, the metabolic state of skeletal muscles is greatly altered. The most drastic changes occur in white muscle, where tuna burn characteristically is found, and involve substrates, intermediates, and end products in energy metabolism. Glycogen and phosphocreatine (PCr) reserves are nearly fully depleted, with lactate and creatine being produced in stoichiometric amounts (Guppy *et al.* 1979; Hochachka 1980). In addition, ATP levels drop and this drop may be presumed to be stoichiometrically represented by an equivalent inosine monophosphate (IMP) accumulation. P_i is also assumed to accumulate in amounts equivalent to PCr depletion. These are standard effects observed in vertebrate white muscle in exhaustive work and their impact has been considered in detail elsewhere (Hochachka 1985a). From the perspective of this paper, the most important point to bear in mind is that the above metabolite concentration changes occur with a net acidification; i.e., H^+ also accumulates as a final metabolic end product which is widely assumed to be the most debilitating because it favors the above undesirable metabolic cascade. With this background, we reasoned that a simple test of the above theoretical burn etiology could be made by a detailed examination of the accumulation of amino acids as end products of acid-mediated proteolysis in damaged *vs* undamaged tuna white muscle.

Materials and methods

Samples of burned and unburned regions of the epaxial musle of yellowfin tuna were obtained from the commercial fishermen at Kewalo Basin, Honolulu. Weighed amounts of each sample were diced, then extracted in 0.6 M perchloric acid. The amino acid content and composition of each sample was then determined using a Beckman Amino Acid Analyzer as described elsewhere (Mommsen *et al.* 1980).

Results

The concentrations of all amino acids detectable in tuna muscle acid extracts are shown in Table 1. While the qualitative composition of the amino acid pool is not at all unusual for fish white muscle (Mommsen *et al.* 1980; Guppy 1978), the total pool size (averaging about 30–35 μmol g^{-1}) is some three fold higher than found in salmon under conditions of protein mobilization (Mommsen *et al.* 1980). However, by tuna standards, the pool size in these samples is actually smaller than in healthy unstressed individuals, mainly because of large depletions of histidine which obviously occur postmortem. Histidine levels in these samples are in the 30 μmol g^{-1} range, compared to values some three fold higher in unstressed tuna (Abe *et al.* 1986).

Three additional points need emphasis: Firstly, whereas the generally elevated levels of amino acids are consistent with overall proteolysis occurring in tuna white muscle post mortem, no major differences are detectable between burnt and control sampling sites. The cause of burn, therefore, must be more subtle than the mere presence or absence of general proteolysis. Secondly, the pattern obtained is grossly different from that found in fish muscle which is actively mobilizing protein as a carbon and energy source (Table 2; also see Mommsen *et al.*

Table 1. Acid-extractable amino acid profile in burnt tuna white muscle compared to control (unburnt) samples taken from the same animals, expressed in nmoles g^{-1} wet weight of tissue

Amino acid	Concentration*	
	Control	Burnt
taurine	560.7 (trace–988)	509 (23–855)
aspartate	trace	trace
threonine	636 (237–832)	608 (311–936)
serine	733 (594–804)	809 (572–1092)
asparagine	trace	trace
glutamate	1559 (1227–1979)	1534 (877–2409)
glutamine	334 (128–450)	400 (133–900)
proline	110 (trace–188)	225 (trace–458)
glycine	1666 (696–2470)	1726 (650–3005)
alanine	1718 (941–2217)	1719 (933–2572)
citrulline	trace	trace
valine	1094 (812–1364)	1152 (1006–1360)
methionine	212 (174–265)	187 (139–216)
isoleucine	508 (329–619)	492 (398–670)
leucine	837 (713–907)	838 (691–980)
tyrosine	261 (150–366)	263 (169–329)
phenylalanine	229 (119–335)	193 (104–303)
ornithine	359 (223–520)	409 (315–464)
lysine	2382 (1555–2863)	2198 (1663–2625)
histidine	19929 (16202–23508)	17889 (13587–22812)
3-methyl-histidine	trace	trace–92
arginine	226 (163–271)	336 (244–466)
anserine	52080 (44979–55995)	51313 (48395–52992)

*Average of three samples, with range of values given in brackets below. Muscle samples were graded as slightly, moderately, or heavily burnt, but since in any given animal these did not differ from control values in the same animal, all data are pooled. In individuals showing heavily burnt regions, dominant, metabolically active amino acids (each as glutamate, glycine, and alanine) occurred at 2–3 higher fold concentrations in control and burnt samples than they did in similar samples from individuals showing less severe burn damage. Thus we conclude that this difference simply represents differing amounts of generalized proteolysis (perhaps due to longer post-mortem periods prior to sampling) and does not relate directly to the etiology of burn.

Table 2. Several metabolically active amino acids in burnt and control tuna muscle (expressed as mole fraction of total amino acid pool and compared to fish muscle that is actively mobilizing protein)

Amino acid	Unburnt control	Burnt muscle	*In vivo* fish muscle metabolizing protein*
aspartate	0.2	0.2	1–4
glutamate	4.7 (3.6–5.4)	4.7 (2.5–6.7)	7
glycine	5.0 (2.0–6.7)	5.3 (1.9–8.3)	10
alanine	5.2 (2.7–6.9)	5.4 (2.7–7.2)	20–30
lysine	7.0 (5.3–8.3)	6.8 (6.3–7.7)	3–7
histidine	59.7 (54.9–68.1)	55.6 (48.1–66.8)	3–36
arginine	0.7 (0.5–0.8	1.0 (0.8–1.3)	2–3

*From Mommsen *et al.* (1980). The similarity between unburned control and burned samples again supports the conclusion that generalized proteolysis is similar in both regions; the difference between them seems to reside mainly in the disassembly of myofilaments in burned mytomal regions. The marked difference between post-mortem muscle and fish muscle actively metabolizing protein as a fuel is assumed to reflect a loss of metabolic regulation of proteolysis in post-mortem muscle.

1980), which might be anticipated if the post-mortem proteolysis was not under metabolic control; in fish actively utilizing protein as a fuel, proteolysis is clearly closely regulated (Hochachka and Somero 1984). Thirdly, the absence of significant levels of 3-methyl-histidine is a most significant result of these analyses (Tables 1 and 3). This animo acid occurs only in the white muscle isoforms of myosin and in actin and is so accurate a marker (Tonomura 1973) that its release rate is utilizable as a means for quantifying the rate of breakdown of these proteins (Lowell *et al.* 1986). In our samples, 3-methyl-histidine is found at modest levels only in burned muscle (Tables 1 and 3); its occurrence at low levels in most of our extracts means that myosin and actin are **not** hydrolyzed to any appreciable degree under capture and initial boat-storage conditions.

Table 3. Observed and theoretical maximum levels of 3-methyl-histidine in burnt tuna muscle

Burn condition	3-methyl-histidine	Approx. theoretical maximum*
slight	trace	500
moderate	trace	500
heavy	92	500

*Assuming complete hydrolysis of myofilaments. The white muscle isoform of myosin is assumed to occur at 0.1 μmol per g muscle and contain two moles of 3-methyl-histidine per mol myosin; actin occurs at about 0.3 μmol per g muscle and contains one mol of 3-methyl-histidine per mol actin (Tonomura 1973).

Discussion

The protease paradox

In considering the above information, two conclusions can be made with confidence. On the one hand, breakdown of tissue texture (in the extreme, to a seemingly self-digested muscle) implies a breakdown of myofibers in tuna burn. On the other hand, the absence of 3-methyl-histidine in appreciable quantities emphatically rules out extensive hydrolysis of myosin and actin, the two dominant proteins of muscle fibers. In trying to resolve this paradox, it is necessary to retrace our steps to reconsider what is currently known of mechanisms in cell death and in subsequent proteolysis. In the process, new insights are gained on the etiology of tuna burn.

Decoupling metabolic and membrane functions

From recent analyses of this problem (Hochachka 1986) it is evident that O_2-lack and a consequent drop in ATP synthesis rates initiates an autocatalytic cascade leading to cell damage and cell death (Fig. 1). Most animal tissues under these conditions are unable to maintain a balance between the rates of ATP hydrolysis required by ion pumps and rates of ATP synthesis achievable by energy metabolism. Metabolic and membrane functions consequently become decoupled, a process manifest in a large K^+ efflux, followed by a Na^+ influx, as ICF and ECF pools move towards equilibrium positions. Dropping ATP level, by favoring opening of K^+ channels (Spruce *et al.* 1985), may be the metabolic signal which initiates this membrane-metabolism decoupling. Be that as it may, when the membrane potential drops sufficiently, voltage-dependent Ca^{++} channels are opened which lead to a large influx of ECF Ca^{++}, a process favored by a very large ECF – ICF concentration gradient, and leading to increasing $[Ca^{++}]$ in the cytosol. Increased Ca^{++} levels in the cytosol are also favored by increased Na^+/Ca^{++} exchange at the cell membrane, and possibly at the mitochondria, as well as by Ca^{++} leakage from the sarcoplasmic reticulum (SR).

Although Ca^{++} at low concentrations plays many pivotal functions in cell regulation and signal transduction, at high concentrations it is a known intracellular toxin, (see Hochachka 1985b for literature). Under conditions of tuna capture and boat-storage, we propose that one of its most debilitating actions occurs via the activation of Ca^{++}-dependent proteases in muscle. The literature on Ca^{++}-activated proteases is vast and we cannot review it all here (see Zeman *et al.* 1985). Suffice to emphasize that for some half-decade now it has been known that these proteases preferentially and selectively degrade specific components of the contractile machinery: troponin, tropomyosin, C-protein, and M-protein (Bird *et al.* 1980). Later studies show Ca^{++}-induced weakening of Z-discs (Hattori and Takahashi 1982) and a stringent requirement for Ca^{++} in the subsequent complete removal of Z-lines and α-actinin by Ca^{++}-activated neutral proteases (Reddy *et al.* 1983). If uncontrolled, as may be expected in tuna burn, these effects of course are severely damaging and they are exacerbated by proteinases acting on the SR so as to increase Na^+/Ca^{++} exchange and thus further add to the ICF Ca^{++} pool (Phillipson and Nishimoto 1982).

The net effect of Ca^{++}-activated protease function is the disassembly of myofilaments prior to any significant degradation of myosin or actin (Bird et al. 1980). As this is exactly what seems to be observed in tuna burn, we propose that the above protease paradox can be fully resolved on the as-

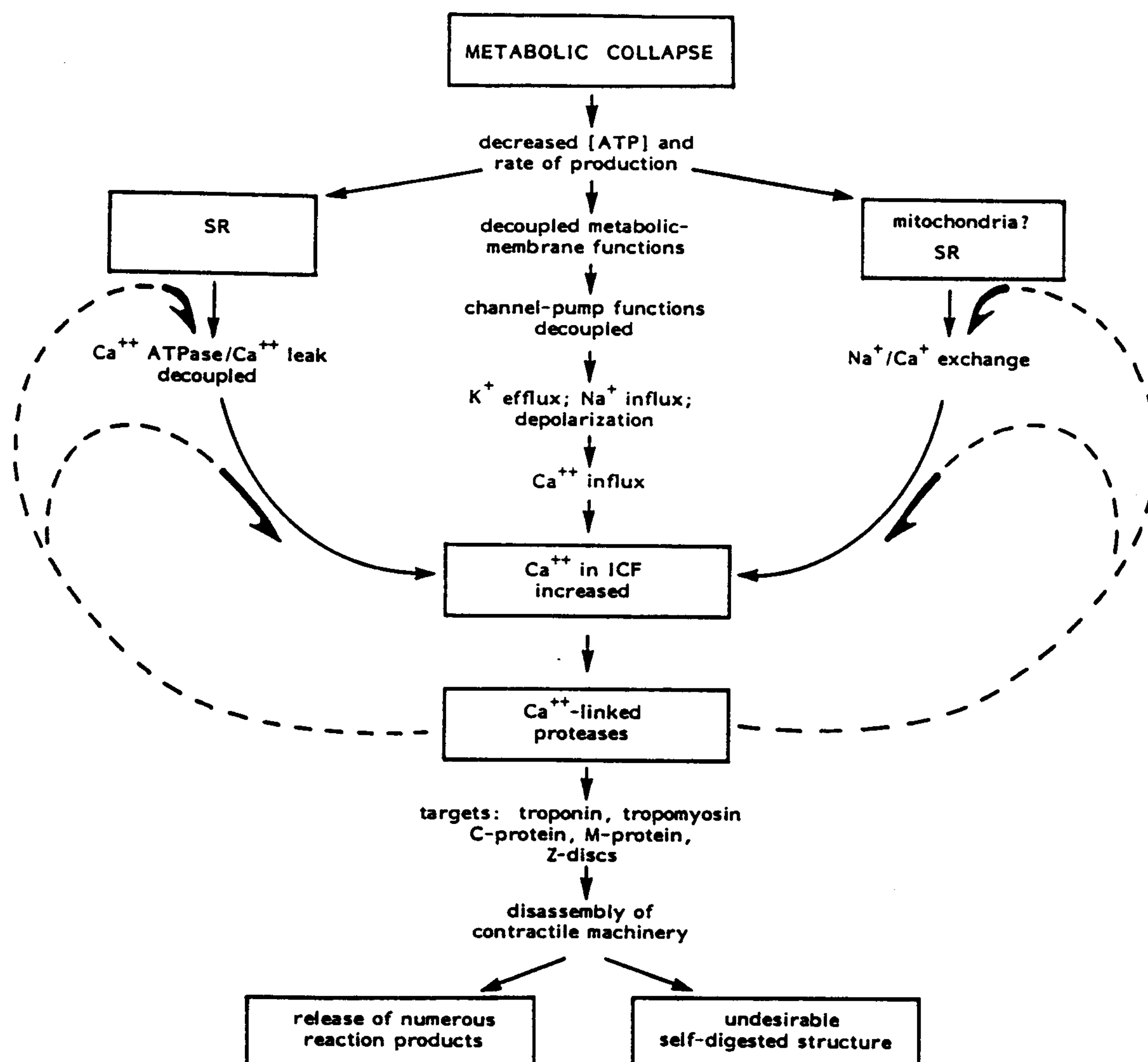

Fig. 1. Proposed model of metabolic events initiated during online capture of tuna and terminating in burnt muscle in post-mortem tuna.

sumption that Ca^{++}-dependent protease function is the initial tissue-damaging step in the development of burn.

The metabolic scenario emerging from this analysis, therefore, is the following (see Fig. 1):

metabolic collapse (due initially to O_2 lack) → membrane collapse (K^+ efflux) → rise in Ca^{++} ICF concentration (from ECF, SR, and mitochondrial pools) → proteolysis of minor structural components; disassembly of myofilaments → undesirable texture development prior to actual hydrolysis of myosin and actin

Models such as this one are useful if they allow explanations of previously perplexing data and if they lead to testable predictions. With respect to the former criterion, our model satisfactorily explains

the main paradoxical finding of this study: development of a soft, self-digested muscle texture in the absence of myosin and actin hydrolysis (in the absence of significant amounts of 3-methyl-histidine accumulation (Table 3)). Additionally, the model better explains why self-digested texture can appear in the absence of severe acidosis: Ca^{++}-dependent proteases typically display near-neutral pH requirements while the classical lysosomal proteases considered the most likely candidates for proteolysis in earlier burn studies require very acidic conditions (about pH 4.5) for activity (see Bird *et al.* 1980; Zeman *et al.* 1985, for further literature).

In this context, it may be useful to mention that previous workers interested in tuna muscle deterioration have placed especial focus on end product accumulation, in particular lactate and H^+ accumulation. The reason for such emphasis, of course, was the implicit assumption that low pH is required for extensive tissue proteolysis. However, we now feel that such emphasis may be premature and perhaps unjustified. Whereas glycogen and lactate are utilized and accumulated in stoichiometric amounts, accumulation of H^+ is not stoichiometric with the accumulation of lactate because much of the protons produced are effectively absorbed by intracellular buffers. Aside from proteins, tuna white muscle typically has two additional sources of very effective intracellular buffers: phosphate which accumulates under these conditions, and histidine plus histidine related compounds (anserine and carnosine), which in total are found in tuna white muscle at over 100 μmol g^{-1} (Abe *et al.* 1985, 1986). Even in post-mortem muscle samples these total about 70 μmol per g tissue (Table 1). Thus, while extracellular pH may drop to pH 5.5–pH 6 within a few hours of capture of the animal (Cramer *et al.* 1981), intracellular H^+ concentrations may or may not mimic this pattern, and thus pH_i values may not fall this low. Nevertheless, no one would argue that the pH_i probably is well below pH 7; a value of pH 6–6.5 is not unreasonable based on known end products and buffering components (Abe *et al.* 1985). This value is consistent with Ca^{++}-protease function, but inconsistent with extensive lysosomal tissue degradation. Thus our model can readily explain a previously perplexing situation – proteolysis without drastic acidification.

In addition to helping us understand features of tuna burn that are already known, our model makes several specific, and highly testable predictions. Perhaps the most useful (i.e. easily measurable) of such predictions are the following:

(i) K^+_{ECF} concentrations in burnt tuna muscle should exceed values in control nonburnt samples.

(ii) Ca^{++}_{ICF} concentrations in burnt tuna muscle should exceed values in control nonburnt samples.

(iii) Troponin/tropomyosin breakdown intermediates should occur in higher concentration in burned muscle than in unburned samples.

(iv) Troponin or tropomyosin concentrations in burnt tuna muscle should be less than in nonburnt control samples; in severely burnt muscle, even myosin and actin hydrolysis should occur (Pemrick and Grebenau 1984).

(v) In the event of (iv) above, the concentration of 3-methyl-histidine (from actin and myosin) in severely burnt tuna muscle should exceed that in nonburnt control samples. (This result is implied in Table 3 but needs reconfirmation with larger sample numbers).

Finally, in our view, since the degenerative cascade process probably begins during online capture, the main difference between burnt tuna muscle and nonburnt post-mortem muscle is in how far along the autocatalytic, self-destructive pathway (of Fig. 1) each tissue region has progressed. The difference between burnt and normal post-mortem tuna muscle may be quantitative and perhaps less drastic than previously supposed.

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