Biochimica et Biophysica Acta, 1144 (1993) 235–248

BBABIO 43893

Review

The secretory-vesicle ascorbate-regenerating system: a chain of concerted H^+/e^--transfer reactions

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(Received 8 March 1993)

Key words: Proton/electron transfer; Secretory vesicle; Ascorbic acid; Cytochrome *b*-561

Contents

I. Introduction

A common misconception is that the individual steps in an electron-transfer chain are necessarily electron-transfer reactions. Many of these reactions involve the transfer of hydrogen atoms (or the concerted transfer of H^+ and e^-) rather than electron transfer alone. This distinction is generally disregarded because H and e^- are considered interchangeable in the aqueous milieu of a cell, but the focus on electrons obscures some general principles underlying the functioning of redox chains. True electron-transfer reactions are not well-suited for redox chains because they are typically non-specific; the favored reaction would be between the initial electron donor and the terminal acceptor bypassing all intermediate electron carriers. Spatial constraints alone cannot account for electron flow through intermediate carriers because, in all redox chains, at least some of the carriers are diffusible. The specificity required for sequentially ordered redox reactions is achieved at least in part by employing hydrogen-atom

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Abbreviations: AH^-, ascorbate monoanion; $A^{\cdot-}$, semidehydroascorbate; DBM, dopamine β-monooxygenase; PAM, peptidylglycine α-monooxygenase; SDAR, semidehydroascorbate reductase.

rather than electron-transfer reactions. H (or concerted H^+/e^-) transfer reactions can be greatly accelerated by enzymatic catalysis, and this permits the specificity essential to the functioning of biological redox chains. The redox chain responsible for regenerating ascorbic acid in secretory vesicles provides a good illustration of this general principle and will be reviewed here from that perspective.

The secretory-vesicle redox chain (Fig. 1) transports reducing equivalents into the vesicles to support intravesicular monooxygenases [1–4]. The monooxygenases oxidize ascorbate to its free radical form, semidehydroascorbate. This intravesicular semidehydroascorbate is reduced back to ascorbate by cytochrome *b*-561, a protein spanning the secretory-vesicle membrane. Cytochrome *b*-561 is reduced in turn by cytosolic ascorbic acid, and the cytosolic semidehydroascorbate thus produced is recycled by semidehydroascorbate reductase in the outer mitochondrial membrane. In this way, reducing equivalents are passed one at a time from semidehydroascorbate reductase, across the secretory vesicle membrane, to the intravesicular monooxygenases. Because the chain passes single reducing equivalents and most of its components are soluble and simple, this system is a good model for studying biological redox chains.

Here, we will review the ascorbate-regenerating system in secretory vesicles, drawing mostly upon studies of the chromaffin vesicles of the adrenal medulla. Because ascorbic acid plays a central role, understanding its properties is essential to comprehending the system. Consequently, we will discuss the properties of ascorbic acid in some detail, arriving at the hypothesis that ascorbic acid, in biological systems, is a donor of single hydrogen atoms. We will consider cytochrome *b*-561 and its reaction with ascorbic acid because it serves as a paradigm for hydrogen atom (or concerted H^+/e^-) transfer between reduced organic compounds and heme proteins. We will review other redox reactions in the secretory-vesicle chain (those catalyzed by dopamine β-monooxygenase and peptidylglycine α-monooxygenase) and examine them applying the perspective of hydrogen-atom transfer. Finally, we shall consider the general implications of hydrogen-atom transfer for biological redox systems.

II. The secretory-vesicle ascorbate-regenerating system

One of the primary biological functions of ascorbic acid is to serve as the reductant for redox reactions occurring within secretory and synaptic vesicles [5,6]. Two reactions, those catalyzed by dopamine β-monooxygenase (DBM) and peptidylglycine α-monooxygenase (PAM), are especially significant. DBM converts dopamine to norepinephrine and mediates catecholamine biosynthesis in adrenal chromaffin cells, in peripheral sympathetic nerve endings and in noradrenergic neurons in the central nervous system [7]. PAM and an associated lyase lead to amidation of the carboxy-termini of many peptide hormones including vasopressin, oxytocin, VIP, neuropeptide Y, α-MSH, substance P, calcitonin and gastrin [8,9]. Ascorbate is, thus, essential for the biosynthesis of two principal classes of intercellular messengers.

Ascorbic acid does not appear to be supplied to the monooxygenases by transport into the secretory vesicles. Ascorbate uptake into chromaffin vesicles, either isolated [3,10–12] or in situ in cultured chromaffin cells [13], is much too slow to support the observed monooxygenase activity. Instead, intravesicular ascorbate is

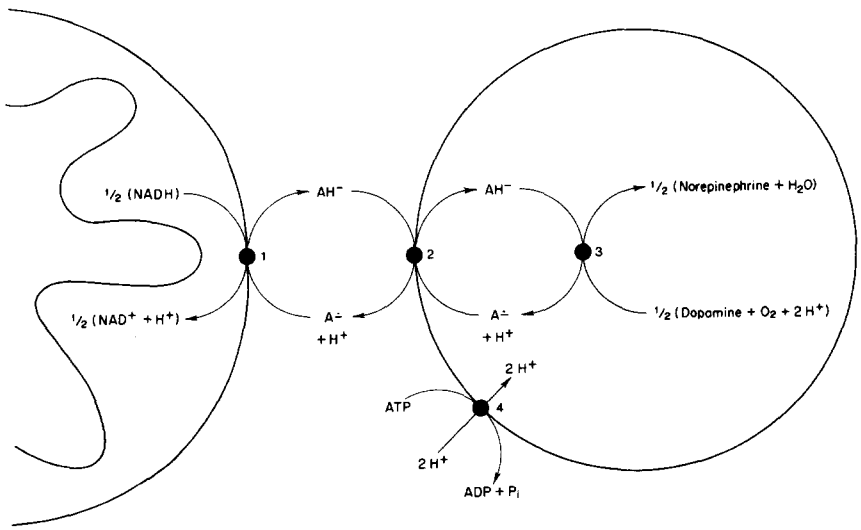

Fig. 1. Mechanism of ascorbic acid regeneration in secretory vesicles. 1, semidehydroascorbate reductase; 2, cytochrome *b*-561; 3, dopamine β-monooxygenase; 4, H^+-translocating ATPase. Reprinted from Ref. 105.

recycled by importing reducing equivalents across the vesicle membrane through cytochrome *b*-561 (Fig. 1). This mechanism, which we proposed in 1983 [1], is now generally [2–4,11,14,15] (although not universally [12, 16,17]) accepted. The evidence for and against it is summarized in the following section.

II-A. Evidence

The ascorbate-regenerating system has been characterized principally using chromaffin vesicles from bovine adrenal medulla. These vesicles contain the catecholamines – epinephrine and norepinephrine – secreted by the chromaffin cells. The vesicles also contain ascorbic acid and the enzyme dopamine β-monooxygenase. Except as noted, the studies described here pertain specifically to bovine chromaffin vesicles. These may be studied either in situ in primary cultures of chromaffin cells, as isolated vesicles containing endogenous enzymes and ascorbate, or as resealed membrane vesicles (ghosts) having membrane-bound DBM and a desired medium trapped inside.

Important features of the ascorbate-regenerating mechanism in secretory vesicles are the following: (1) The monooxygenases oxidize ascorbate to the free radical, semidehydroascorbate. (2) Cytochrome *b*-561 reduces intravesicular semidehydroascorbate. (3) Cytochrome *b*-561 mediates the transmembrane transport of electrons. (4) Cytosolic ascorbate reduces cytochrome *b*-561. (5) Cytosolic ascorbate is regenerated by semidehydroascorbate reductase on the mitochondrial outer membrane. Each of these points has been tested and found to be true.

Production of the ascorbate radical by dopamine β-monooxygenase has been observed using both UV absorbance [18] and an NADH-coupled assay [19]. In addition, DBM activity increases the ascorbate free-radical signal detected by electron paramagnetic resonance in isolated chromaffin vesicles and ghosts [20,21]. That peptidylglycine α-monooxygenase produces semidehydroascorbate has not yet been confirmed. Nevertheless, because PAM shares structural and mechanistic similarities with DBM (Section V), it is likely to produce semidehydroascorbate as well. Moreover, as we shall discuss in Section III, ascorbic acid generally functions as a one-equivalent donor and, in all cases in which the product has been examined, ascorbate-consuming enzymes produce semidehydroascorbate.

That cytochrome *b*-561 reduces semidehydroascorbate is confirmed by the fact that semidehydroascorbate oxidizes the cytochrome. Spectral changes show that the cytochrome is oxidized by external semidehydroascorbate but not by dehydroascorbate [22]. Wakefield et al. [2] showed that DBM activity oxidizes the cytochrome and that this is reversed by semidehydroascorbate reductase. DBM does not oxidize the cytochrome in the absence of ascorbate, suggesting that the oxidation is caused by internal semidehydroascorbate produced by the enzyme. A clear demonstration that the cytochrome is oxidized by internal semidehydroascorbate has been achieved by trapping ascorbate and horseradish peroxidase in chromaffin-vesicle ghosts [23]. Addition of H_2O_2 leads to generation of internal semidehydroascorbate and cytochrome oxidation. The requirement for ascorbate as a mediator between cytochrome *b*-561 and the monooxygenases has been confirmed by the demonstration that the cytochrome cannot donate reducing equivalents directly to either DBM or PAM [24].

Cytochrome *b*-561 mediates the transmembrane transport of electrons. It reacts internally with ascorbate or semidehydroascorbate and externally with ascorbate, semidehydroascorbate or ferricyanide [22,23]. This proves that the cytochrome can mediate redox reactions at both surfaces of the membrane. If the purified cytochrome is reconstituted into liposomes, internal ascorbate can be oxidized by external oxidants [4]. Consequently, cytochrome *b*-561 can act as a transmembrane electron carrier and should equilibrate internal and external pools of ascorbate/semidehydroascorbate.

The argument that ascorbate is the cytosolic reductant of cytochrome *b*-561 is supported by the following arguments: First, ascorbate is the only known endogenous reductant that will reduce cytochrome *b*-561 (Section IV-A). Second, internal ascorbate is consumed by internal DBM activity in the absence but not in the presence of external ascorbate in intact chromaffin vesicles [3,11,15] and in ghosts [14]. Other potential donors including NADH, glutathione, thiourea and homocysteine do not work. Finally, internal semidehydroascorbate, detected by electron paramagnetic resonance, is increased in the presence of external semidehydroascorbate and diminished by external semidehydroascorbate reductase activity [20,21]. These data all present good evidence for a transmembrane redox system using ascorbate as the external donor and semidehydroascorbate as the internal acceptor. These data do not provide direct evidence that this system is cytochrome *b*-561, although the results described previously argue that cytochrome *b*-561 could function in this way. Furthermore, cytochrome *b*-561 is the only heme protein in the chromaffin-vesicle membrane [25]. The possibility that there is another pathway for transmembrane redox flow, and specifically that membrane-bound DBM can accept electrons directly from an external electron donor, has been raised [12,16,17,26]. Reasons for discounting this possibility are discussed in Section V-A.

The final step in the ascorbate regeneration scheme is the reduction of cytosolic semidehydroascorbate by

semidehydroascorbate reductase (SDAR). Diliberto et al. [27] have shown that this enzyme, which uses NADH as the reductant, is present in the outer mitochondrial membranes of chromaffin and other cells. The coupling between SDAR and chromaffin vesicles has been elegantly demonstrated by Wakefield et al. [2,20]. They showed that NADH in vitro reduces cytochrome *b*-561 and intravesicular semidehydroascorbate. By drawing reducing equivalents from NADH, SDAR couples the hydroxylation reaction within the secretory vesicles ultimately to the citric acid cycle within the mitochondria.

II-B. Distribution

Criteria for demonstrating the existence of the ascorbate regenerating system in secretory or synaptic vesicles are (1) presence of ascorbate and an ascorbate-requiring enzyme in the vesicle and (2) presence of cytochrome *b*-561 in the vesicle membrane. These criteria have been met not only for adrenal chromaffin vesicles but also for neurosecretory vesicles from the neurohypophysis. The latter store the peptide hormones oxytocin and vasopressin, and contain ascorbate [28] and peptidylglycine α-monooxygenase [29]. The vesicle membranes also have cytochrome *b*-561 (identified both spectrally and immunologically [30]) and support transmembrane electron flow from internal ascorbate to external ferricyanide or cytochrome *c* [28].

In addition, cytochrome *b*-561 has been identified spectrally in norepinephrine-storing synaptic vesicles from bovine splenic nerve [31] and rat vas deferens [32] and in secretory granules from anglerfish islets of Langerhans [33]. The synaptic vesicles contain dopamine β-monooxygenase and the secretory granules contain the peptidylglycine α-monooxygenase [34,35]. Using antibodies, cytochrome *b*-561 has been localized in a variety of neural and endocrine tissues storing either amidated peptides or catecholamines [30,36]. In particular, the atrium of the heart, the hypothalamus and all three lobes of the pituitary contain both cytochrome *b*-561 [30,36] and PAM [8,37].

There is a report [38] that cytochrome *b*-561 is found in dense vesicles from pig platelets. Using the same spectrophotometric assay, however, we are unable to detect the cytochrome in dense vesicles isolated from human platelets (McKinney, C.D. Özkan, E.D., Zafar, R., Kelley, P.M., Njus, D. and Walz, D., data not shown). Moreover, Duong et al. [30] found no crossreactivity between antiserum against bovine cytochrome *b*-561 and either porcine or bovine platelets. The dense vesicles are not known to store either catecholamines or amidated peptides. Given that these vesicles have no apparent need for cytochrome *b*-561, its presence in them is unlikely.

II-C. Energetics

The ascorbate-regenerating system requires that reducing equivalents flow from the ascorbate/semidehydroascorbate pool in the cytosol to the corresponding pool within the vesicles. Three factors drive this flow in vivo. First, the pH gradient (inside acidic) across the vesicle membrane favors the inward movement of reducing equivalents because the midpoint reduction potential of the ascorbate/$A^{\dot{-}}$ couple is pH dependent [39]. The midpoint potential in the intravesicular space (+0.42 V at pH 5.5) is 90 mV higher than the midpoint reduction potential in the cytosol (+0.33 V at pH 7.0). Second, the membrane potential (inside positive), generated by the proton-translocating ATPase in the vesi-

TABLE I

Rate constants for redox reactions of cytochrome b-561

Rate constants for reaction with internal semidehydroascorbate and external ferricyanide were calculated from the measured rate constants for the reverse reactions using the equation:

$$k_{12} = k_{21} \exp\{(E_2^\circ - E_1^\circ)(F/RT)\}$$

The following values were used for E°: Ferri/ferrocyanide, +0.42 V; Cytochrome *b*-561, +0.14 V [59,65]; Ascorbate/semidehydroascorbate, +0.39 V (pH 6.0), +0.33 V (pH 7.0) and +0.27 V (pH 8.0) [39].

Donor/Acceptor	Side	pH 6.0	pH 7.0	pH 8.0	Ref.
Measured rate constants ($M^{-1}s^{-1}$)					
Ascorbate	In	35 ±12	55 ±12	9.4±3.6	70 (k_0^A)
Ascorbate	Out	310 ±10	450 ±190	570 ±100	69 (k_{-1}^A)
Semidehydroascorbate	Out	$(2.0\pm0.7)\cdot10^6$	$(1.2\pm0.5)\cdot10^6$	$(1.7\pm0.2)\cdot10^5$	69 (k_1^A)
Ferricyanide	Out	$(6.2\pm2.7)\cdot10^5$	$(6.7\pm3.4)\cdot10^5$	$(5.1\pm5.1)\cdot10^4$	70 (k_1^F)
Calculated rate constants ($M^{-1}s^{-1}$)					
Semidehydroascorbate	In	$5.6\cdot10^5$	$8.6\cdot10^4$	$1.4\cdot10^3$	
Ferrocyanide	Out	12.3	13.2	1.0	

cle membrane [40], favors the influx of electrons. Finally, semidehydroascorbate reductase lowers the reduction potential of the cytosolic ascorbate pool by lowering the concentration of cytosolic semidehydroascorbate. The opposite occurs within the vesicles where the monooxygenases raise the potential of the intravesicular pool. This too drives reducing equivalents into the vesicles [2,20].

The behavior of the ascorbate-regenerating system in vivo can be modeled using kinetic data from in vitro studies (see Section IV-B, Table I). For any given rate of intravesicular monooxygenase activity, the flow of reducing equivalents should reach a steady state that will fix the reduction potentials of the cytochrome and the intravesicular and cytosolic ascorbate/$A^{\dot{-}}$ couples. These reduction potentials, estimated as a function of semidehydroascorbate generation rate, are shown in Fig. 2. The maximum rate of dopamine hydroxylation in chromaffin vesicles in situ (measured in the presence of 10 mM dopamine) is approx. 1 nmol/min per mg of chromaffin-vesicle protein [41,42], corresponding to a semidehydroascorbate generation rate of approx. 8 μM/s. This is undoubtedly an unnaturally fast pace, and the normal rate of semidehydroascorbate production is probably about an order of magnitude slower.

The reduction potentials shown in Fig. 2 were calculated assuming that the cytosolic $A^{\dot{-}}$ concentration is held at or below 50 nM by semidehydroascorbate reductase. Given a cytosolic ascorbate concentration of 5 mM [43], this corresponds to a cytosolic reduction potential of +22 mV. The cytochrome does not reach equilibrium with this cytosolic ascorbate pool because of kinetic limitations. The reduction of cytochrome *b*-561 by external ascorbate is relatively slow, because the rate constant is small (Table I) and only a small fraction of the cytochrome is in the oxidized form. Consequently, especially at high rates of monooxygenase activity, the reduction potential of the cytochrome rises considerably above that of the cytosolic ascorbate pool. Internally, the oxidation of cytochrome *b*-561 by semidehydroascorbate is relatively fast, because the rate constant is large (Table I) and most of the cytochrome is in the reduced state. As a result, the internal ascorbate pool is nearly at equilibrium with the cytochrome.

This analysis illustrates the functional logic of the ascorbate regenerating system. In vivo, the cytochrome is maintained in the reduced form, poised to reduce intravesicular semidehydroascorbate with which it reacts quickly. This is precisely the behavior desired in a system intended to scavenge and recycle intravesicular semidehydroascorbate.

The foregoing model was derived without taking the membrane potential into account. The membrane potential set up by the H^+-translocating ATPase should also drive the flow of reducing equivalents into the vesicles, but quantitative evidence for this is still limited. There have been a number of studies showing that outward electron flow creates a membrane potential [1,44] and that inward electron flow dissipates the proton gradient [45]. Two studies show a direct effect of the membrane potential generated by the ATPase

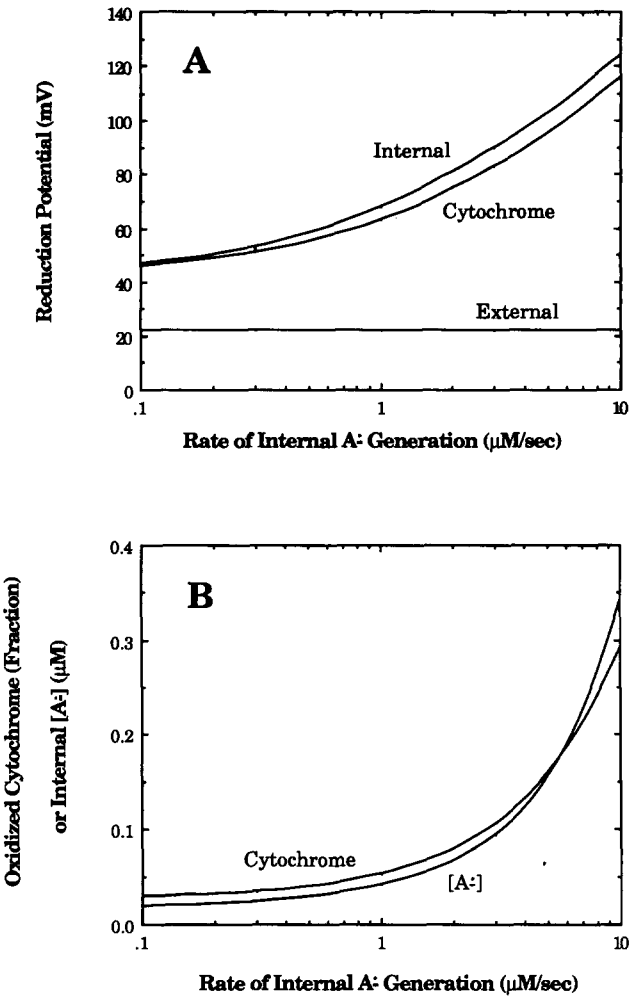

Fig. 2. Reduction potentials of the ascorbate regenerating system as a function of intravesicular monooxygenase activity. The external reduction potential was calculated assuming cytosolic concentrations of 50 nM semidehydroascorbate and 5 mM ascorbate. The cytochrome reduction potential was determined from the ratio [cyt b-561_{ox}]/[cyt b-561_{red}]. This ratio was calculated assuming that the difference between the rate of reduction by cytosolic ascorbate ($0.2855\ s^{-1}\cdot$[cyt b-561_{ox}]) and the rate of oxidation by cytosolic semidehydroascorbate ($7.63\cdot10^{-3}\ s^{-1}\cdot$[cyt b-561_{red}]) equals the rate of internal $A^{\dot{-}}$ generation. Similarly, the internal reduction potential was calculated from the intravesicular semidehydroascorbate/ascorbate ratio taking the intravesicular ascorbate concentration as 22 mM. Internal semidehydroascorbate was calculated assuming that the difference between the rate of $A^{\dot{-}}$ reduction by cytochrome b-561 ($1.25\cdot10^6\ M^{-1}s^{-1}\cdot[A^{\dot{-}}]\cdot$[cyt b-561_{red}]) and the rate of AH^- oxidation by cytochrome b-561 ($35\ M^{-1}s^{-1}\cdot[AH^-]\cdot$[cyt b-561_{ox}]) equals the rate of internal $A^{\dot{-}}$ generation. The intravesicular semidehydroascorbate concentrations and the [cyt b-561_{ox}]/[cyt b-561_{red}] ratios determined in these calculations are shown in Panel B.

on electron flow. Wakefield et al. [2] found that ATP caused a reduction of the cytochrome in the presence of endogenous reductants. In this case, the reduction potential was presumably fixed by the larger external pool of endogenous reductants and the membrane potential (positive inside) drew electrons into the membrane. The extent of reduction was consistent with a 20 mV potential difference between the external medium and the heme. Harnadek et al. [23] fixed the reduction potential within the ghosts by trapping ascorbate, H_2O_2 and horseradish peroxidase. In this case, ATP increased oxidation of the cytochrome by drawing electrons from the membrane back to the internal redox pool. The extent of oxidation indicated a potential difference of 20 mV between the heme and the internal space. Taken together, these results are consistent with a membrane potential of 40 mV and a heme located midway through the membrane. Two cautions should be noted, however. First, although the ATP-dependent membrane potential is typically about 40 mV in ghosts [40], it was not measured in either of the above studies. Second, the above interpretation assumes that the midpoint potential of the cytochrome is independent of the membrane potential.

III. Properties of ascorbic acid

Although ascorbic acid (vitamin C) was first isolated over half a century ago, its biological actions are still controversial and its chemical behavior is still not widely understood. The biological effects of ascorbic acid can be explained, however, if we accept that ascorbic acid functions as a donor of single hydrogen atoms under physiological conditions. This means that the two-step oxidation of ascorbic acid to its fully oxidized product, dehydroascorbate, is rare. It also means that the one-step oxidation to the free radical semidehydroascorbate involves the loss of a hydrogen atom (or the concurrent loss of a proton and an electron) rather than the loss of an electron alone. We will present the thermodynamic arguments supporting this perspective, and explore the ramifications of viewing ascorbic acid as a donor of single hydrogen atoms.

III-A. Oxidation states of ascorbic acid

The unique redox properties of ascorbic acid are a consequence of its molecular structure and the oxidation and protonation states it can assume. These species of ascorbic acid, along with the pK values and reduction potentials characterizing the equilibria between them, are shown in Fig. 3.

The structure of the fully oxidized form, dehydroascorbate, is crucial to the behavior of ascorbic acid. The structure (A) that would be formed by the two-equivalent oxidation of ascorbate is highly strained having three adjacent carbonyl groups in a five-membered ring (Fig. 3). This form is not detectable in aqueous solution. Instead, dehydroascorbate is found in a less strained bicyclic form [46]. The highly strained structure of dehydroascorbate represents a substantial energetic barrier to the complete two-equivalent oxida-

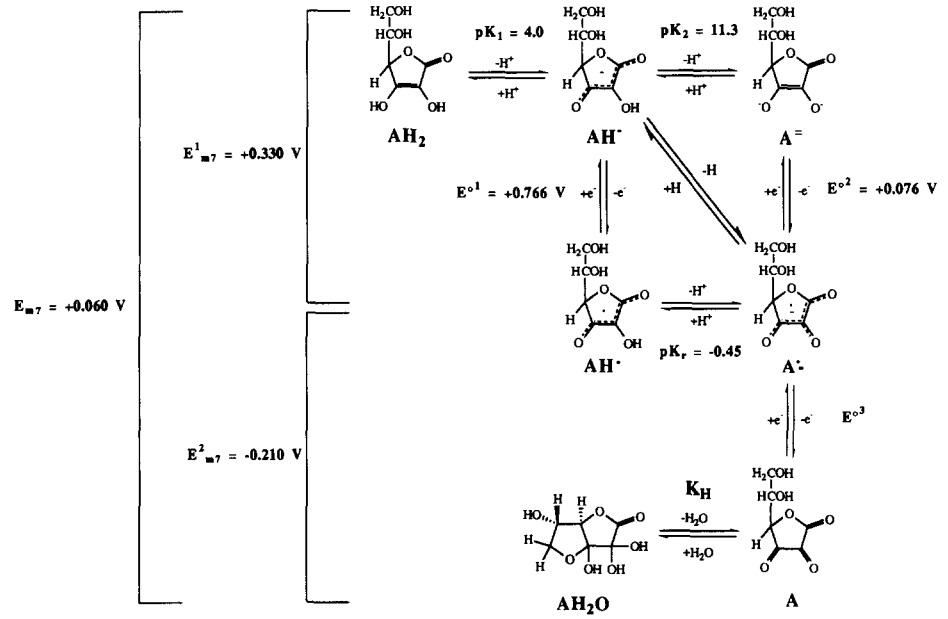

Fig. 3. Interconversion of ascorbate species. Protonation reactions are shown horizontally and electron-transfer reactions are shown vertically. Abbreviations for ascorbate species are: AH_2, ascorbic acid; AH^-, ascorbate monoanion; A^{2-}, ascorbate dianion; $A^{\cdot-}$, semidehydroascorbate anion; A, dehydroascorbate; AH_2O, hydrated dehydroascorbate. Values for pK and reduction potentials are taken from Njus and Kelley [54]. Williams and Yandell [52] and Creutz [47] have presented similar compilations.

tion of ascorbate [47]. Although not widely appreciated, this has the following consequences: (1) Ascorbate tends to donate one reducing equivalent rather than two. (2) Semidehydroascorbate is relatively unreactive being a rare example of a free radical that is a poorer electron donor than its parent compound.

The ascorbate free radical, semidehydroascorbate ($A^{\cdot-}$), is stabilized by its capacity to distribute the unpaired electron over a number of atoms. It has a pK of -0.45 [48], so the protonated form is virtually non-existent under physiological conditions. Semidehydroascorbate disproportionates quickly exhibiting strict second-order kinetics. The rate constant is strongly pH-dependent, and kinetic evidence suggests that the reaction involves the transient formation of a dimer [49]. Rearrangement within the dimer probably circumvents formation of the high-energy tricarbonyl structure of dehydroascorbate and allows the disproportionation reaction to occur. Disproportionation is crucial because it eliminates the free radical keeping the reduction potential of the ascorbate/semidehydroascorbate couple down in the neighborhood of 0 to $+0.1$ V under normal physiological conditions.

Ascorbic acid itself exists in protonated (AH_2), monoanionic (AH^-) and dianionic (A^{2-}) forms. The pK values are 4.04 and 11.34 [50], so the monoanion is predominant at physiological pH. The monoanion is not a good electron donor, however, because it has a relatively high midpoint reduction potential. The dianion is a much more powerful electron donor but it represents an exceedingly small fraction of ascorbate at physiological pH [51,52]. Cytochrome c and molecular oxygen are both reduced by the ascorbate dianion but not by the monoanion [51,53]. Thus, these reactions are both strongly pH-dependent, occurring readily at higher pH but much more slowly under neutral or acidic conditions.

III-B. Ascorbate is a biological donor of single hydrogen atoms

At physiological pH, both ascorbic acid and semidehydroascorbate are present predominantly in their anionic forms. This means that the transition between them involves the net gain or loss of a hydrogen atom. As a consequence, ascorbate works best, in the physiological pH range, as a donor of single H atoms. Ascorbate is a poor electron donor because the monoanion has a very high reduction potential ($E° = +0.766$ V) and the dianion has an insignificant concentration.

Its ability to donate single hydrogen atoms accounts for the effectiveness of ascorbic acid as a free radical scavenger [54]. The most common biological free radicals ($OH^{\cdot}$, $ROO^{\cdot}$, $R^{\cdot}$, etc.) are missing an H atom rather than an electron. Consequently, they are acceptors of single H atoms and are reduced back to their parent compounds (H_2O, ROOH, RH) most effectively by a donor of single hydrogen atoms. As an example, the tocopheroxyl radical is reduced back to tocopherol (vitamin E) most effectively by hydrogen atom transfer from the ascorbate monoanion (Fig. 4).

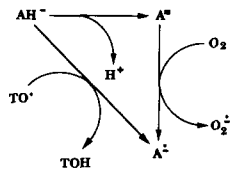

Fig. 4. Electron transfer and hydrogen-atom transfer reactions of ascorbic acid. The ascorbate dianion (A^{2-}) reduces oxygen to superoxide by electron transfer. The ascorbate monoanion (AH^-) reduces the tocopheroxyl radical ($TO^{\cdot}$) to tocopherol (TOH) by hydrogen atom transfer.

The fact that it is a donor of single hydrogen atoms rather than of electrons also allows ascorbic acid to be relatively stable and innocuous in the presence of O_2. Low-potential electron donors readily reduce molecular oxygen to the very destructive superoxide radical. Although ascorbate has a sufficiently low reduction potential, it reduces O_2 rather slowly at physiological pH [53]. The rate increases markedly at higher pH, indicating that it is the ascorbate dianion that reduces O_2 to $O_2^{\cdot-}$ (Fig. 4). The ascorbate monoanion, because it is a hydrogen atom donor, and not a good electron donor, tends not to reduce O_2 to superoxide.

As a substrate for redox enzymes, ascorbate acts as a good donor of single reducing equivalents. Peroxidase [55], ascorbate oxidase [56], dopamine β-monooxygenase [18,19] and cytochrome b-561 all use ascorbate in this way and form semidehydroascorbate. Interestingly, these enzymes use ascorbate as a reducing agent quite effectively at neutral and even slightly acid pH, where the concentration of ascorbate dianion should be exceedingly small. As we will show below, kinetic data suggest that ascorbate reacts with cytochrome b-561 by losing the equivalent of a single hydrogen atom. We have termed the mechanism concerted proton/electron transfer.

IV. Cytochrome *b*-561

IV-A. Structure and properties

Cytochrome b-561 (30061 Da) has recently been cloned and sequenced [57]. Hydropathy analysis suggests that the protein may have six transmembrane domains with both amino and carboxy-termini being on the cytoplasmic side of the membrane (Fig. 5). The cytochrome is not glycosylated but does have covalently bound fatty acid [58]. The location and nature of the

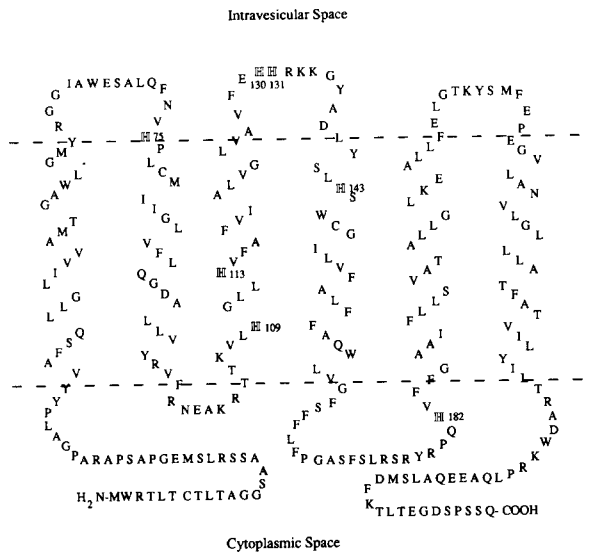

Fig. 5. Primary structure of bovine cytochrome *b*-561. Histidine (H) residues are highlighted and numbered. Similar topological diagrams are discussed by Perin et al. [57] and by Fleming and Kent [63].

fatty acid linkage are not yet known, but the fatty acid can be removed by hydroxylamine.

Cytochrome *b*-561 is believed to contain only one heme. In a very careful study, Apps et al. [59] used the pyridine hemochromogen method to quantitate heme and Western blotting to quantitate the apoprotein. They found a cytochrome/heme stoichiometry of 1.09. The cytochrome purified by Wakefield et al. [60] had an extinction coefficient (reduced-oxidized) of 21 000 $M^{-1} cm^{-1}$ at 561 nm relative to 575 nm. These results, as well as the fact that the reduced-oxidized spectrum does not change shape as cytochrome *b*-561 is titrated, would all suggest the presence of only one heme. This is in dispute, however. Based on a non-linear Nernst plot, circular dichroism data and low-temperature EPR, Degli Esposti et al. [61] suggested that there are two hemes in cytochrome *b*-561.

It is likely that the fifth and sixth ligands for the heme iron are histidine residues. Methionine as a sixth ligand is unlikely, because cytochrome *b*-561 does not have the characteristic weak absorption band at approx. 700 nm [62]. There are seven histidine residues in bovine cytochrome *b*-561. The sequence around His 109 has homology to sequences around heme-liganded histidines in other *b*-cytochromes. Therefore, Degli Esposti et al. [61] and Fleming and Kent [63] have suggested that His-109 and His-182 are ligands for a heme near the cytoplasmic surface of the membrane. Degli Esposti et al. placed a second heme between His-75 and His-143 near the intravesicular surface. At present, most of the evidence is consistent with a single heme possibly nearer the cytoplasmic face of the protein. This is suggested by the fact that the cytochrome is reduced more quickly from the cytoplasmic side than from the intravesicular side (Table I).

Cytochrome *b*-561 is a high potential *b*-type cytochrome and is reduced by ascorbate and dithionite but not by NADH, NADPH, glutathione, ferrocyanide or durohydroquinone [64–67]. It is oxidized by ferricyanide, cytochrome *c* and semidehydroascorbate but not by dehydroascorbate [22,66]. The midpoint reduction potential (+0.140 V) is independent of pH, but redox titration yields a non-linear Nernst plot ($\log\{[Cyt_{ox}]/[Cyt_{red}]\}$ vs. E_h) [59,61,65].

IV-B. Concerted proton / electron transfer

The kinetics of the reactions between cytochrome *b*-561 and ascorbate and semidehydroascorbate have been studied in some detail [68–70]. The original objective of these studies was to test the kinetic feasibility of the ascorbate regeneration mechanism. More recently, it has become apparent that this approach may yield more general insights into biological redox reactions; in particular, it has given us a deeper understanding of the behavior of ascorbic acid and has suggested the hypothesis that enzymes which use ascorbic acid as a reducing agent react with the ascorbate monoanion via a mechanism involving concerted proton/electron transfer.

The simplest kinetic analysis assumes that the rate of a reaction is proportional to a rate constant and to the concentrations of the two reactants. We know that this is an oversimplification in this case, because reduction of cytochrome *b*-561 by external ascorbate exhibits Michaelis-Menten kinetics and saturates at high ascorbate concentrations [65]. Nevertheless, the rate constant approach minimizes the number of parameters that must be measured and gives a good first approximation of the behavior of the system at subsaturating ascorbate concentrations. Consequently, we have determined rate constants for a number of reactions as summarized in Table I. The details of the measurements are given elsewhere [68–70].

These kinetic measurements have the following implications: First, as mentioned above, ascorbate reduces the cytochrome more rapidly from the outside of the secretory vesicle than from the inside. This is consistent with the heme being located closer to the cytosolic surface of the membrane. Second, internal semidehydroascorbate is reduced by cytochrome *b*-561 much faster than it disproportionates. Given an intravesicular semidehydroascorbate concentration of 50 nM, the rate of reduction should be $3.5 \cdot 10^{-6}$ M/s at pH 6.0 (the concentration of cytochrome *b*-561 is approx. 130 μM relative to the intravesicular volume). By contrast, the rate of semidehydroascorbate disproportionation will be an order of magnitude less: $1.25 \cdot 10^{-7}$ M/s [49].

The final implication of the rate constants is that the reactions of cytochrome *b*-561 with ascorbate and semidehydroascorbate are too fast to be consistent with 'outer-sphere' electron-transfer. Cytochrome *c*, for example, is reduced by ascorbate at a relatively slow rate at physiological pH (Fig. 6). Moreover, this rate is highly pH-dependent, increasing by a factor of approx. 10 with every unit of pH. This is consistent with reduction of cytochrome *c* by the ascorbate dianion [51]. By contrast, the rate of cytochrome *b*-561 reduction is not very pH-dependent on either surface of the membrane (Fig. 6). Furthermore, cytochrome *b*-561, which has a midpoint reduction potential 120 mV lower than cytochrome *c*, should be reduced by ascorbate much more slowly. Instead, it is reduced more rapidly especially at the external surface. The rate of oxidation of cytochrome *b*-561 by semidehydroascorbate is also faster than expected when compared to the rate of oxidation of cytochrome *b*-561 by ferricyanide [70]. Again, this suggests that the reaction between cytochrome *b*-561 and semidehydroascorbate occurs too quickly to be attributed to outer-sphere electron transfer.

The rapid reaction rates between ascorbate/semidehydroascorbate and cytochrome *b*-561 can be rationalized in terms of a mechanism involving concerted proton/electron transfer [70]. According to this scheme, cytochrome *b*-561 converts the abundant ascorbate monoanion directly to the semidehydroascorbate anion by extracting both an electron and a proton (Fig. 7). This reaction, therefore, is not kinetically limited by the low abundance of the ascorbate dianion.

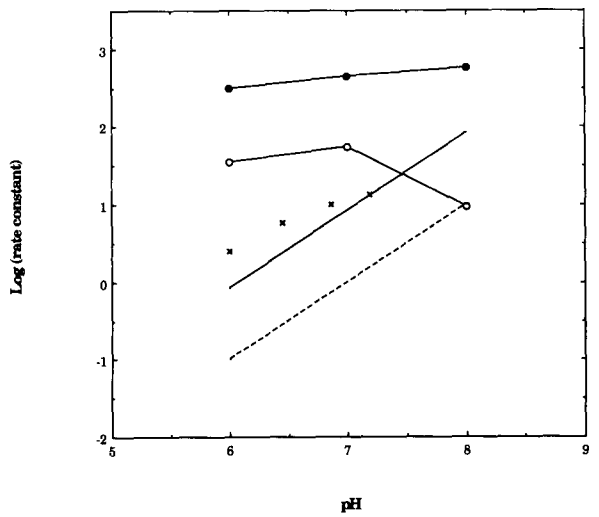

Fig. 6. Comparison of rates of reduction of cytochrome *b*-561 and cytochrome *c* by ascorbate. Rate constants for cytochrome *c* reduction are from Yamazaki [106] (×) and Al-Ayash and Wilson [51] (———). Rate constants for cytochrome *b*-561 reduction are from Table I: (○), inside; (●), outside. The theoretical rate constant was calculated from the rate constant for cytochrome *c* reduction using Marcus theory [70] (---).

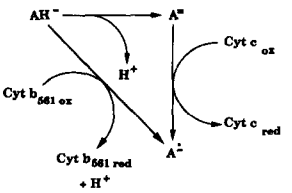

Fig. 7. Electron transfer and concerted H^+/e^- transfer reactions of ascorbic acid. Cytochrome *c* is reduced by electron transfer from the ascorbate dianion (A^{2-}). Cytochrome *b*-561 is reduced by concerted H^+/e^- transfer from the ascorbate monoanion (AH^-).

Moreover, the rate can be much less pH-dependent. Concerted proton/electron transfer will also facilitate the oxidation of cytochrome *b*-561 by semidehydroascorbate because the product is the ascorbate monoanion instead of the energetically unfavorable ascorbate dianion.

Concerted proton/electron transfer is a consequence of the fundamental principle that ascorbic acid is most effective at physiological pH as a donor of single hydrogen atoms. This means that the cytochrome must be structured so as to facilitate hydrogen atom transfer or its equivalent even though only the electron is needed to reduce the heme iron.

IV-C. Ascorbate binding sites

The idea that the reaction between ascorbate and cytochrome *b*-561 occurs by concerted proton/electron transfer suggests the existence of an active site. Cytochrome *b*-561 would be expected to have two such sites, one internal and one external. Indeed, there is now good evidence for an external ascorbate binding site. Flatmark and Terland [65] originally showed that the rate of cytochrome *b*-561 reduction saturates at high concentrations of external ascorbate, exhibiting a K_m of about 340 μM. This is consistent with an observation by Dhariwal et al. [42] that the K_m for external ascorbate for in situ norepinephrine synthesis is 280–290 μM. We have found that treatment of cytochrome *b*-561 with diethylpyrocarbonate inhibits reduction by external ascorbate, and this inhibition is reversed by hydroxylamine (Kelley, P.M. and Njus, D., data not shown). This indicates that a histidine residue is involved in the reaction.

How does the binding site catalyze concerted proton/electron transfer? A proton from ascorbate could be transferred to a group on the protein as electron transfer to the heme occurs. Alternatively, a cationic group in the binding site could promote the dissociation of the second proton from bound ascorbate stabilizing the ascorbate dianion in the reaction complex. Either mechanism would poise bound ascorbate for electron transfer to the heme.

If concerted proton/electron transfer is indeed a mechanism for making a hydrogen atom donor compatible with an electron acceptor, then it creates a potential problem: superoxide generation. When the reductant (ascorbate) is in the active site, it is poised for electron donation to the heme. If the heme is already reduced, the ascorbate may donate e^- to any electron acceptor. In particular, molecular oxygen is readily available for reduction to the highly destructive superoxide radical. To protect against this, does the reduced cytochrome not bind ascorbate? Does the reduced cytochrome not hold ascorbate in a configuration poised for electron donation? Or is the cytochrome site simply structured to prevent access by molecular oxygen? These are questions yet to be resolved.

V. Intravesicular monooxygenases

Secretory and synaptic vesicles contain two known monooxygenases: dopamine β-monooxygenase (DBM) and peptidylglycine α-monooxygenase (PAM). These enzymes share several similarities: both require copper for full functionality, both require ascorbate as a reductant and both reduce one atom of molecular oxygen to water while incorporating the other into their respective substrates [6–8]. There may also be similarities in reaction mechanism, such as the passage of the substrate through a radical intermediate. Both enzymes have been reviewed elsewhere [7–9]. Here we will focus on the manner in which reducing equivalents are passed from ascorbate to the enzymes.

V-A. Dopamine β-monooxygenase

Dopamine β-monooxygenase catalyzes the hydroxylation of dopamine to yield norepinephrine (Fig. 8). The protein is a tetramer and exists in both soluble and membrane-bound forms. The soluble form has four identical monomers with a molecular mass of 70–73 kDa. The membrane-bound form has two subunits of the soluble type and two slightly larger subunits (75–77 kDa). cDNA clones for the human [71,72], bovine [73–75], rat [76] and mouse [77] enzymes have been isolated and sequenced. The homology among the different proteins is between 70 and 80%. A single mRNA codes for both soluble and membrane-bound subunits [71], and the soluble form is thought to be derived from the membrane-bound form. The conversion mechanism and the nature of the membrane linkage are controversial, however. The N-terminal signal sequence contains the only potential transmembrane domain prompting speculation that the uncleaved signal sequence could anchor DBM to the membrane. However, Taylor et al. [78] have reported that the N-terminal sequence of the membrane-bound subunit does not correspond to the signal sequence. Covalent attachment to lipid anchors is also possible, but none have yet been detected [79].

Dopamine β-monooxygenase contains eight copper atoms per tetramer [80,81] and uses ascorbate as a one-equivalent donor [18,19]. Ascorbate must reduce the enzyme before the other substrates (molecular oxygen and dopamine) bind [82]. Reduction of the copper atoms, of course, will require electrons rather than hydrogen atoms. The pH optimum of DBM is between 5 and 6 [83], consistent with reduction by the ascorbate monoanion. This would suggest that DBM oxidizes the ascorbate monoanion by concerted proton/electron transfer in a manner analogous to cytochrome *b*-561. DBM has a K_m for ascorbate of 1.25 mM, and its activity is inhibited when a histidine residue is modified by diethylpyrocarbonate [84]. Again, like cytochrome *b*-561, DBM appears to have a histidine-containing ascorbate-binding site that mediates concerted proton/electron transfer. While not definitive,

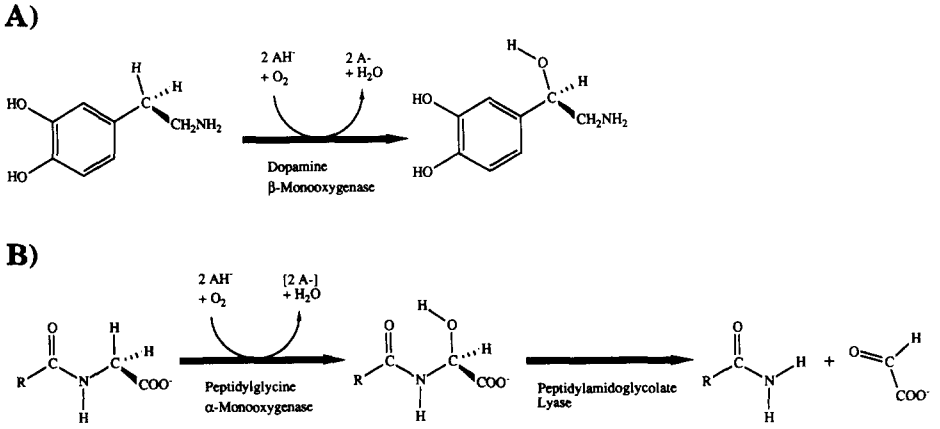

Fig. 8. Mechanisms of intravesicular redox reactions. (A) The reaction catalyzed by dopamine β-monooxygenase leading to the biosynthesis of norepinephrine and epinephrine. (B) The reactions catalyzed by the PAM system leading to the amidation of peptide hormones.

these results suggest that concerted H^+/e^- transfer might be a general mechanism for reaction between ascorbate and metalloproteins.

There continues to be speculation that membrane-bound DBM can accept electrons directly from a cytosolic reducing agent [12,16,17]. Kent and Fleming [24] tested this possibility using DBM reconstituted into proteoliposomes and obtained negative results. Nevertheless, the observation that external ferrocyanide stimulates internal DBM activity has led some to conclude that there must be another pathway (not cytochrome *b*-561) for transmembrane electron flow [26]. Ahn and Klinman [16] hypothesized that membrane-bound DBM accepts electrons directly from an external electron donor and that an internal aqueous mediator (ascorbate) is not needed.

The rate of hydroxylation is relatively slow, however, and the stimulation caused by external ferrocyanide can be rationalized in terms of cytochrome *b*-561-mediated electron flow. Grouselle and Phillips [26] found that 250 μM external ferrocyanide stimulated the rate of intravesicular tyramine hydroxylation by approx. 3 nmol/min per mg of ghost protein. Ahn and Klinman [16] reported that external ferrocyanide (0.2–2 mM) stimulates the hydroxylation of intravesicular dopamine by about 4 nmol/min per mg. The rate constant for reduction of cytochrome *b*-561 by ferrocyanide is 13.2 $M^{-1}s^{-1}$ (Table I) or 1.8 μl/min per mg of membrane protein. Since the reduction potential will approach the midpoint potential of ferri/ferrocyanide (+0.42 V), the cytochrome will be nearly completely oxidized, and the rate of electron flow from 1 mM ferrocyanide to cytochrome *b*-561 will be approx. 1.8 nmol/min per mg. This slow rate is insufficient to cause net reduction of cytochrome *b*-561, but it is enough to account for the observed stimulation of dopamine β-monooxygenase activity.

Stewart and Klinman [17] have also reported that extravesicular ascorbate supports DBM activity, and they argue that the electrons are imported into the vesicle through the uncleaved signal sequence holding the membrane-bound DBM subunits to the membrane. Given the amino-acid sequence of the putative transmembrane domain, this is improbable. First, the signal sequence of the bovine enzyme [73–75] lacks any histidine or cysteine residues. This excludes the possibility of any metal binding sites in the transmembrane domain that might serve as a pathway for transmembrane electron transfer. Second, at most a dozen amino acids on the amino-terminus might be exposed to the cytosol [73]. It seems quite unlikely that they could function as an ascorbate binding site. Again, the absence of any histidine residues argues against a site capable of mediating concerted proton/electron transfer. Finally, as mentioned, there is evidence that the signal sequence does not remain on the N-terminus of membrane-bound DBM [78]. Given that the machinery for transmembrane electron transfer is present in cytochrome *b*-561 and not apparent in dopamine β-monooxygenase, the concept of direct donation of cytosolic reducing equivalents to intravesicular monooxygenase must be considered highly improbable.

V-B. Peptidylglycine α-monooxygenase and peptidylamidoglycolate lyase

The activity responsible for amidating peptide hormones was discovered a little over a decade ago [29], but progress in elucidating the mechanism has been rapid (for reviews, see Refs. 8 and 9). The realization in the last few years that the activity is actually catalyzed by a two-enzyme system has clarified a number of mysteries.

The amidating system acts on a glycine-extended peptide and removes the C-terminal carboxyl and α carbons releasing them as glyoxylate (Fig. 8B). Using a synthetic substrate, Bradbury et al. [29] were able to show that the amide nitrogen is derived from a glycine residue. This led to the identification [85,86] and later purification of a peptidylglycine α-amidating monooxygenase [87]. Surprisingly, the pH optimum of the purified monooxygenase for the amidation reaction was much higher than the internal pH of the secretory vesicles [87,88]. This raised some controversy as to the real function and mechanism of this enzyme [89]. The controversy was resolved only recently by the discovery that a protein fraction from the same tissue could stimulate amidation by the monooxygenase at low pH [88,90]. This led to the realization that the amidation reaction is actually catalyzed by two separate enzymes [91–94], the peptidylglycine α-monooxygenase (PAM) and peptidylamidoglycolate lyase (PGL). The monooxygenase catalyzes the hydroxylation of the glycine-extended peptide to produce the peptide-α-hydroxyglycine [95]. The subsequent cleavage reaction, which splits off glyoxylate, will proceed spontaneously at high pH, but the lyase is needed to catalyze the reaction at low pH. The optima for both the monooxygenase and the lyase are in the pH 4.5–5.5 range, coinciding with the internal pH of the secretory-vesicle matrix.

Because the amidating enzymes are present only in very small amounts even in the pituitary, the molecular biological approach has been especially helpful in their characterization. The monooxygenase actually occurs in two different forms. The larger, designated A, is approx. 54 kDa, and is converted to B (about 38 kDa) by proteolysis [9,87]. The peptidylamidoglycolate lyase is encoded in the same gene as the two monooxygenases and has an apparent molecular mass of 45–53 kDa [92,94,96].

The hydroxylation reaction catalyzed by the monooxygenase is the step that requires ascorbate, copper

and molecular oxygen. The peptidylglycine α-monooxygenase has some mechanistic similarities to dopamine β-monooxygenase [97–99], including cofactor requirements [7,8,86] and inhibition [100]. Like DBM, PAM hydroxylates in a stereospecific manner [97 101,102] and has a broad substrate specificity [103]. Since PAM is similar to DBM in terms of mechanism, it is probable that ascorbate reduces the copper atoms in PAM. The low pH optimum, however, indicates that the monooxygenase is using the ascorbate monoanion as its reductant and so is taking the equivalent of a hydrogen atom from ascorbate. This argues that peptidylglycine α-monooxygenase, like dopamine β-monooxygenase and cytochrome *b*-561, converts the ascorbate monoanion to the semidehydroascorbate anion by concerted proton/electron transfer.

VI. Redox traffic control: some rules of the road

In the secretory-vesicle redox chain, ascorbic acid functions as a mediator carrying reducing equivalents between enzymes. Cytoplasmically, it couples mitochondrial semidehydroascorbate reductase with vesicular cytochrome *b*-561. Intravesicularly, it couples the cytochrome with the monooxygenases, dopamine β-monooxygenase and peptidylglycine α-monooxygenase. In both compartments, ascorbic acid exists predominantly as the monoanion and functions as a donor of single H atoms. Consequently, in order to react with ascorbate, the enzymes must be able to convert the ascorbate monoanion to the semidehydroascorbate anion by catalyzing the equivalent of H atom transfer. We have termed this process concerted proton/electron transfer.

There are adaptive reasons for the evolutionary selection of this kind of mechanism. A mediator carrying electrons would react nonspecifically by outer-sphere electron transfer [104]. By contrast, H atom carriers permit enzymatic specificity. A particular carrier, therefore, will not interfere with other redox systems or with other steps in the same redox chain. Diversion of reducing equivalents through undesirable side reactions will also be minimized. Of particular significance here is the reduction of molecular oxygen to the superoxide radical, a reaction that is typical of low-potential electron donors but not of hydrogen atoms donors of equal potential.

The principles elaborated for ascorbic acid apply to other organic reducing agents as well. Quinones and flavins, for example, differ from ascorbate in that they undergo a two-equivalent rather than a one-equivalent transition. Nevertheless, they can both transfer one equivalent at a time, forming a free-radical intermediate. It can be argued that, for both of these compounds, the transition between the fully reduced and free-radical forms should involve H atom transfer or the equivalent. Both quinones and flavins when fully reduced exist predominantly in the neutral form (RH_2) at physiological pH. The free-radical species are neutral ($RH^{\cdot}$) or anionic (R^-). Therefore, electron donation yielding the radical cation ($RH_2^{+\cdot}$) is not energetically favored. Hydrogen atom transfer from RH_2 yielding $RH^{\cdot}$ is preferred. Biological selection arguments apply in these cases as well. Since quinones and flavins are low-potential reducing agents, it is important that they not react spontaneously with other carriers farther down the redox chain or with endogenous oxidants in the cell. In particular, if either quinones or flavins donated single electrons rather than single H atoms, they would reduce O_2 to superoxide. As a consequence, the reduced compounds are likely to react with metalloenzymes by the equivalent of H-atom transfer rather than by electron transfer. It seems reasonable to expect then that metalloenzymes using these substrates as cofactors will have an active site capable of mediating concerted proton/electron transfer.

The restriction against electron transfer is not quite so stringent for the second step in the oxidation of flavins and quinones. The free-radical intermediates should be short-lived and sequestered for their lifetime in an active site. Their chances of reacting with stray electron acceptors are thus more remote. The uncharged fully oxidized forms (R) could be indiscriminate electron acceptors; they have low midpoint potentials so the likelihood of encountering an undesired but sufficiently strong electron donor is quite low. The chemical behavior of quinones and flavins also suggests that the proscription against electron transfer may not apply to the second step. The pK values of the radicals are near neutrality, so the uncharged ($RH^{\cdot}$) and anionic ($R^{\dot{-}}$) forms are comparable in concentration and energy. Therefore, the transition between the fully oxidized form and the free radical could occur by either hydrogen atom (R to $RH^{\cdot}$) or electron (R to $R^{\dot{-}}$) transfer.

The discussion above is intended to illustrate some general considerations and not to define mechanisms for particular reactions involving quinone or flavin cofactors. To generalize further, these are not the only two examples to which these principles may be extrapolated. In every living cell, countless vital processes depend upon redox reactions involving organic substrates and metalloenzymes. In each of these, similar considerations apply. Reactants that transfer electrons alone tend to be avoided because their reactivity is non-specific. Enzymes, therefore, probably do not catalyze just a simple electron transfer reaction. Many may work by providing hydrogen atom donors a way to yield a proton as well as an electron, a phenomenon which we have termed concerted proton/electron transfer. For these enzymes, the secretory-vesicle

ascorbate-regenerating system, a chain of one-equivalent redox reactions, is an excellent, simple model.

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