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Adrenalin Autoxidation and Cu_2Zn_2 Superoxide Dismutase

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Superoxide dismutase is known to specifically inhibit adrenalin autoxidation. In contrast noradrenalin and dihydroxyphenylalanine autoxidation reactions are inhibited by 20% only. To account for this phenomenon the formation of a specific ternary complex between oxidizing adrenalin and the copper–zinc protein was deduced from extensive circular dichroism measurements. Contrary to observations with the latter catecholamines, characteristic changes of the chiroptical properties of both adrenalin in the presence of oxygen and the protein moiety of superoxide dismutase were seen. The changes of optical properties could not be observed, when the enzyme devoid of its active site or the partially reconstituted apoprotein employing either zinc or copper, reacted with adrenalin. This suggested the histidine-bridged copper–zinc active site to be essential for the protein's specific reactivity. This conclusion is supported by 360 MHz proton nuclear magnetic resonance measurements, which revealed an induced chemical shift of histidine resonances by the adrenalin–superoxide dismutase binding.

X-ray photoelectron spectrometric measurements support another aspect of the specific inhibitory action of superoxide dismutase. An unusual low nitrogen 1s-electron binding energy of adrenalin compared to other catecholamines can be explanatory for the fact that borate inhibited adrenalin cannot prevent the formation of the ternary complex with the enzyme. Therefore binding of adrenalin to the protein via *the ethanol-amine side chain must be made responsible for an altered autoxidation pathway in the presence of superoxide dismutase.*

Introduction

The detection and evaluation of both superoxide dismutase activity and the generation of superoxide radicals using the adrenalin autoxidation assay has gained considerable interest [1, 2]. Due to its convenient application, this method is frequently employed in homogenates [3], blood [4], milk [5], and tumours [6]. However, there are dramatic differences in the reactivity of superoxide dismutase–active copper chelates including the protein and low molecular weight copper complexes. The latter copper chelates usually remain inactive or accelerate the autoxidation [7, 8], while superoxide dismutase copper is a marked inhibitor [2, 8]. In contrast no such difference is seen in the pulse radiolytically determined catalysis of superoxide dismutation [9–12]. In order to explain this phenomenon a possible complex between superoxide dismutase and the substrate, being in a transient excited state, was proposed [8, 13].

To provide molecular aspects for the explanation of this ternary complex a more detailed study was desired. Circular dichroitic properties of the substrate, the partially reconstituted apoprotein and the native enzyme devoid of its active site were compared. High resolution proton 360 MHz nuclear magnetic resonance spectrometry proved useful to monitor the C(2)- and C(4)-protons of histidine residues of superoxide dismutase [14, 15], of which six of the eight residues per subunit constitute the active site [16]. Chemical shifts of the histidine proton resonances should indicate changes of the active site geometry in the course of the adrenalin–superoxide dismutase binding. It is suggested that the nitrogen in the side chain of the substrate plays a major role in this adrenalin–enzyme complex, since borate-inhibited adrenalin was also reactive [8]. Prior to adrenochrome formation the side chain nitrogen is involved in the rate-limiting step leading to the indole compound [17]. Differences in the electron density on the nitrogen atoms were expected. Thus the nitrogen 1s-electron binding energy values of adrenalin, noradrenalin and dihydroxyphenylalanine were measured by X-ray photoelectron spectrometry.

Experimental

Reagents

All catecholamines were commercially available products of analytical grade purity. They were obtained from EGA Chemie, Steinheim, Ferak, Berlin,

Merck, Darmstadt, Serva, Heidelberg and Sigma, München. α-methyl-noradrenalin was a gift from Hoechst, Frankfurt.

Bovine erythrocyte Cu_2Zn_2-superoxide dismutase was isolated following the procedure commonly used in this laboratory [18]. The protein was freed from metals by the gel-filtration technique and in the presence of 10 m*M* ethylenediaminetetraacetic acid at pH 3.8 [19]. Partial reconstitution of the apoprotein was accomplished by adding 5% stoichiometric excesses of either $ZnCl_2$ or $CuCl_2$. After 24 h incubation the protein solutions were concentrated in a cylindrical collodion bag (Sartorius, Göttingen, SM 13 200) under reduced pressure (1600 Pa). Extraneous bound metals were removed chromatographically (Sephadex G-25) [20]. Destruction of the active site of superoxide dismutase was achieved by 1 h ultraviolet irradiation (35 W, type 5012 Sterisol, Hanau) of a 0.2 m*M* protein solution in a 1 cm light path quartz cell at 24 °C.

Autoxidation of Catecholamines

The incubation conditions for the autoxidation measurements at 25 °C were: 850 μl 0.1 *M* carbonate buffer pH 10, 100 μl water or protein additions to give the final concentrations desired, and 50 μl 10 m*M* catecholamine stabilized with HCl at pH 2. The reactions were started by adding catecholamine. The formation of coloured oxidation products was registered by a Pye Unicam SP 1800 spectrophotometer at 480 nm (adrenalin) and 455 nm (noradrenalin and dihydroxyphenylalanine).

Spectrometry

Metal concentrations were determined on an atomic absorption spectrometer (Perkin Elmer, 400 S, Überlingen) equipped with the graphite furnace HGA 76 B. Protein solutions were measured without further treatment. Protein concentrations were calculated by the known absorption coefficients of superoxide dismutase ($\epsilon_{259} = 9840\ M^{-1}\ cm^{-1}$ [18]) and aposuperoxide dismutase ($\epsilon_{259} = 3670\ M^{-1}\ cm^{-1}$ [20]).

Circular dichroism was monitored on a J 20 A spectropolarimeter (Jasco, Tokio). Cylindrical quartz cells of variable light path in the range 1–20 mm were employed.

X-ray photoelectron spectra were recorded at 24 °C employing the commercially available catecholamines. These were applied to the adhesive side of cellotape (Scotch tape, 3M Co.) fitted onto a cylindrical aluminium sample holder. The carbon signal (1s at 284.0 eV) of the aliphatic hydrocarbons on the adhesive side of the cellotape served as internal standard. Binding energies were measured by analyzing kinetic energies of electrons emitted from the sample during $Mg_{k\alpha_{1,2}}$ -irradiation (1253.6 eV) at 3×10^{-4} Pa. Spectra were recorded on a Varian VIEE-15 spectrometer furnished with a Varian 620 L computer, under the following conditions: work function 6.0 eV, analyzer energy 100 eV, sweep width 20 eV, sweep time 20 s, number of channels 200 and 10 scans. All spectra were time averaged and smoothed by a least squares method, enhancing the reproducibility to better than ±0.1 eV.

360 MHz proton nuclear magnetic resonance spectra were obtained from a Bruker Spectrospin 360 unit combined with an 8 T superconducting magnet (Oxford Instruments Ltd.) and computer accessory to perform convolution difference calculations [21]. Incubation 70 h prior to nuclear magnetic resonance measurement of lyophilized superoxide dismutase samples in 99.6% ^2H_2O ascertained complete ^2H-replacement of exchangeable protons. After repeated lyophilysation samples were dissolved in 99.6% ^2H_2O for measurement. The proton signal of the vacuum grease silicone served to calibrate chemical shifts. pH adjustments were monitored with a meter (Knick, model 645, Berlin) taking into account the solvent isotope effect [22].

Results

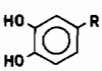
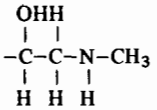
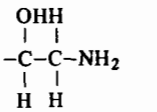
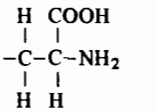
Autoxidation of Catecholamines

The autoxidation of adrenalin in 0.1 *M* carbonate buffer at pH 10 proceeded at rates of 0.028 ΔA_{480}/min identical with earlier data [2]. The rates of autoxidation of noradrenalin and dihydroxyphenylalanine were considerably faster with 0.033 ΔA_{455}/min and 0.045 ΔA_{455}/min respectively, leading to absorption maxima (plateau) after 15–20 minutes. Adrenalin autoxidation resulted in maximal absorption within 8–10 minutes followed by an absorption decrease before melanization caused slow absorption increase after 20 minutes of the reaction. In contrast to adrenalin, autoxidation reactions of the other catecholamines were much less inhibited by the addition of 0.1 μ*M* superoxide dismutase concentrations (Table I).

Circular Dichroism

Circular dichroism of adrenalin in the presence of μ*M* or less superoxide dismutase concentrations (negligible contributions of the protein to Cotton effects: $\theta < 0.5$ [degree]) differs markedly from adrenalin alone (Fig. 1). After twenty minutes the protein-inhibited autoxidation monitored at 480 nm reaches its maximum [23, 24]. At this stage adrenalin autoxidation products exert Cotton bands at 290 nm ($[\theta]_{290} = 300$ [degree·cm^2·decimol^{-1}]), at 330 nm ($[\theta]_{330} = 340$ [degree·cm^2·decimol^{-1}]). Added superoxide dismutase gave rise to the formation of a new Cotton effect at 400 nm ($[\theta]_{400} = -400$ [degree·cm^2·decimol^{-1}]) and an increase with considerable blue shift of the Cotton band at 330 nm to

TABLE I. Autoxidizing Catecholamines in the Presence of Superoxide Dismutase.[a]

Catecholamine 	R	Rate of autoxidation of 0.5 m*M* solution at pH 10 $\Delta A/min$ (λ)	Inhibition of the autoxidation by 100 n*M* SOD* %	Concentration of SOD* at which inhibition is detectable n*M*
Adrenalin		0.028 (480nm)	95	0.5
Noradrenalin		0.033 (455 nm)	19	50
Dihydroxyphenylalanine		0.045 (455 nm)	22	10

*SOD = Cu_2Zn_2 superoxide dismutase. [a]Autoxidation conditions at 25 °C were: 850 μl 0.1 *M* carbonate buffer pH 10, 100 μl water or protein additions to give the indicated final concentrations, 50 μl 10 m*M* catecholamine stabilized with HCl at pH 2. Reactions were started by the addition of catecholamine. Formation of coloured oxidation products was registered by a Pye Unicam SP 1800 spectrometer.

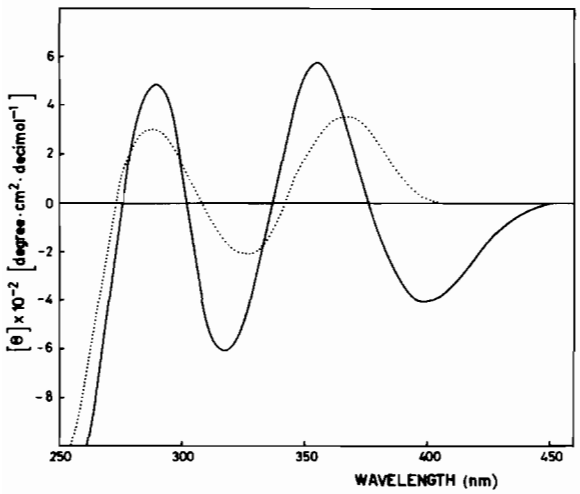


Fig. 1. Circular dichroism of autoxidizing adrenalin. 0.5 m*M* adrenalin (....) after the addition of 1 μ*M* superoxide dismutase (——) in 0.1 *M* carbonate. Spectra were taken after 20 minutes of the reaction using a 1 cm light path quartz cell. Incubation conditions were exactly as described in legend to Table I.

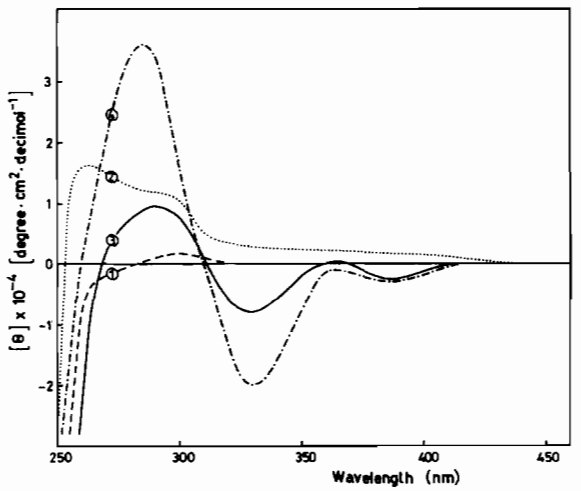


Fig. 2. Circular dichroism of partially reconstituted aposuperoxide dismutases. 50 μ*M* Zn_2-aposuperoxide dismutase ①, 50 μ*M* Cu_2-aposuperoxide dismustase ② in 0.1 *M* carbonate buffer pH 10.50 μ*M* Zn_2-aposuperoxide dismutase in the presence of 0.5 m*M* adrenalin ③, and 50 μ*M* Cu_2-aposuperoxide dismutase in the presence of 0.5 m*M* adrenalin ④. Spectra were taken in 1 cm light path quartz cuvettes after 10 minutes of autoxidizing adrenalin.

317 nm ($[\theta]_{317}$ = −600 [degree·cm²·decimol⁻¹]) and at 370 nm to 355 nm ($[\theta]_{355}$ = 580 [degree·cm²·decimol⁻¹]) is observed. Neither oxidizing noradrenalin nor oxidizing dihydroxyphenylalanine showed similar alterations of their oxidation products in the presence of superoxide dismutase. Throughout, the observed chiroptical effects were identical with those of the autoxidizing substrate itself.

The formation of these new Cotton bands confirms the previous conclusion of the formation of a ternary complex of superoxide dismutase in the

presence of oxidizing adrenalin [8]. In order to elucidate the role of the active site metals during this ternary complex formation the dichroic changes of partially reconstituted aposuperoxide dismutase in the presence of adrenalin were examined.

The Zn_2-aposuperoxide dismutase lacks any chromophore and the circular dichroism is consistent with earlier observations; the spectrum was essentially the same as obtained from the apoprotein (Fig. 2) [25]. Comparing the conformational changes of the ternary complex of superoxide dismutase and oxidizing adrenalin [8] with those of the Zn_2-aposuperoxide dismutase–adrenalin complex only one positive Cotton band at the position of the shoulder at 290 nm ($[\theta]_{290} = 9\,600$ [degree·cm^2·decimol^{-1}]) is seen. The positive 360 nm band appears to be leveled off while the negative band at 384 nm appears more distinct at 390 nm ($[\theta]_{390} = -2\,300$ [degree·cm^2·decimol^{-1}]).

The Cu_2-aposuperoxide dismutase exhibits Cotton bands identical with those of native superoxide dismutase, however, of reduced magnitude (Fig. 2) ($[\theta]_{260} = 16\,000$ [degree·cm^2·decimol^{-1}], $[\theta]_{300} = 10\,400$ [degree·cm^2·decimol^{-1}], and $[\theta]_{360} = 2\,400$ [degree·cm^2·decimol^{-1}]). In the presence of oxidizing adrenalin only one marked positive Cotton band is seen at 285 nm ($[\theta]_{285} = 36\,000$ [degree·cm^2·decimol^{-1}]) lacking the shoulder at 295 nm of the superoxide dismutase–adrenalin complex [8]. The negative Cotton extremum at 325 nm is red-shifted by 5 nm and dramatically increased ($[\theta]_{330} = -20\,000$ [degree·cm^2·decimol^{-1}]) compared to $[\theta]_{325} = -3\,800$ [degree·cm^2·decimol^{-1}]. Again no positive band is detectable at 360 nm while the negative band at 390 nm is more pronounced than with the native protein.

The circular dichroism of the fully reconstituted apoprotein in the presence of oxidizing adrenalin was identical with that reported for native superoxide dismutase [8]. In the 200–250 nm region no conformational changes were observed by added adrenalin in either the native and fully reconstituted protein or the partially reconstituted apoproteins. Obviously the circular dichroism of the adrenalin–superoxide dismutase-complex in the 250–300 nm region results from the addition of the spectra obtained from the Zn_2- and Cu_2-apoprotein in the presence of adrenalin.

Destruction of the histidine ligands around the metals of the active site was expected to reveal as to which degree the native state of the active site of superoxide dismutase is essential for the observed ternary complex formation. After irradiation of the enzyme with ultraviolet light for 1 h [26] the breakdown of the chromophores in the visible region and diminished Cotton bands between 260–320 nm are seen (Fig. 3). Upon prolonged irradiation only negligible changes of the spectrum were detectable ($[\theta]_{280} = 14\,800$ [degree·cm^2·decimol^{-1}] and $[\theta]_{475} =$

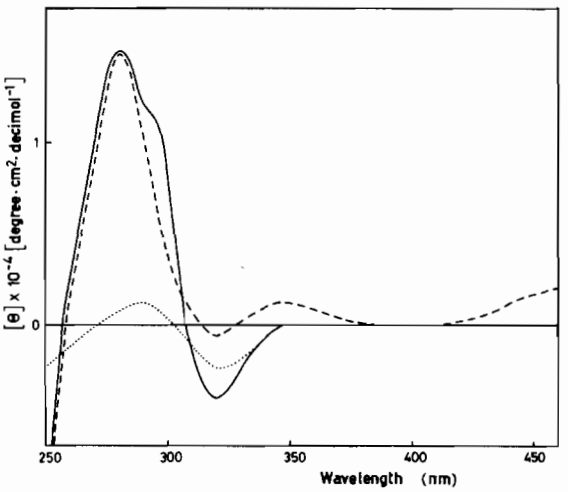


Fig. 3. Circular dichroism of superoxide dismutase devoid of its active site. 50 μM denatured superoxide dismutase (– – – –), in the presence of 0.5 mM adrenalin (——), and of 0.5 mM adrenalin alone (....). Spectra were recorded after 10 minutes of the autoxidation reaction in 0.1 M carbonate buffer pH 10. Incubation conditions as in legend to Table 1. 1 h ultraviolet irradiation (35 W, type 5012, Sterisol lamp, Hanau) was sufficient to destroy the active sites of superoxide dismutase.

2 000 [degree·cm^2·decimol^{-1}]). In the presence of adrenalin the conformation of the denatured protein remains unaffected. The circular dichroism spectrum represents the superposition of the spectra of adrenalin oxidation products over the protein spectrum. No specific interaction of adrenalin with the inactivated protein is apparent.

360 MHz Proton Nuclear Magnetic Resonance

360 MHz nuclear magnetic resonance spectrometry combined with convolution difference calculations resolves proton resonances of superoxide dismutase. As the aim was to detect alterations of the active site geometry of the enzyme during adrenalin binding, only a chemical shift region of 5–10 ppm needs to be monitored (Fig. 4). In this region proton resonances are due to aromatic residues and non-exchanged NH-protons, the latter appearing between 8–10 ppm. The remaining resonances are assigned to four phenylalanines, one tyrosine and eight histidine residues per subunit of superoxide dismutase. Chemical shift studies revealed a pH-dependence of only two resonances in the apoprotein and the reduced protein, and are considered in the following assignments of proton resonances at pH 10. Tyrosine resonances appear as a doublet at 6.5 ppm and 7.0 ppm and aromatic proton resonances of phenylalanine overlap histidine C(4)-proton resonances in the region of 6.5–7.5 ppm, while histidine C(2)-proton resonances are clearly shifted downfield to 7.5–9.0 ppm. The present spectra are exceedingly

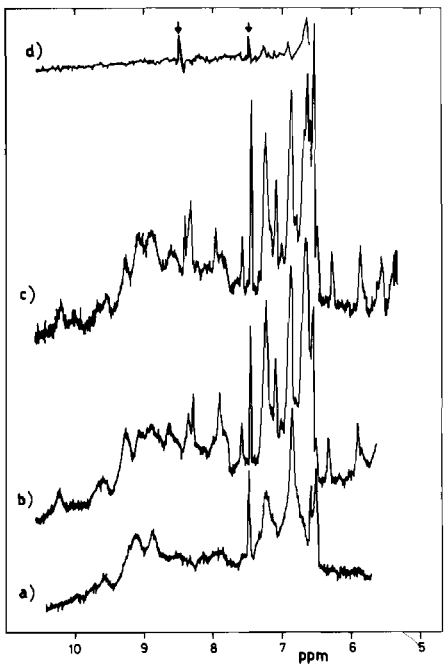


Fig. 4. 360 MHz proton nuclear magnetic resonance of the low field region of deuterated superoxide dismutase: a) 1 m*M* Cu(II)-protein in 50 m*M* carbonate pH 10 (corrected); b) 1 m*M* Cu(I)-protein in 50 m*M* carbonate pH 10 (corrected); c) superoxide dismutase as under a) and 1 m*M* adrenalin in 50 m*M* carbonate pH 10. d) calculated difference spectrum (1 m*M* superoxide dismutase and 3 m*M* adrenalin in 50 m*M* carbonate) – spectrum c). Lyophilized protein samples were incubated in 99.6% ^2H_2O 70 h prior to measurement. Lyophilized superoxide dismutase was dissolved in ^2H_2O to yield 300 µl test volumes of the desired concentrations. The pH was adjusted using ^2HCl taking into account the solvent isotope effect. Superoxide dismutase solutions were reduced using excess dithionite. Convolution difference spectra were calculated scanning the spectrum 1000 times at 23 °C.

well resolved and the general features are in accordance with earlier data obtained on a 270 MHz spectrometer [14].

A spectrum of superoxide dismutase in the presence of autoxidizing adrenalin (c) matches the spectrum of the reduced protein (b). Adrenalin protons at pH 10 cause resonances at 6.6–6.9 ppm (aromatic ring protons), between 3.05 ppm and 3.20 ppm (methyl protons of the side chain), and 2.6 ppm (N-methyl protons) and hence do not interfere with proton resonances of histidine residues. Besides the reduction of the protein-copper by the interaction with adrenalin, an increase of resonance intensities is observed (c). In order to detect whether proton transfer to superoxide dismutase is caused by adrenalin a difference spectrum (ratio adrenalin: enzyme = 3:1–1:1) was calculated (d). Thus an increase of resonance intensity of a histidine C(4)-proton at 7.45 ppm and a slight shift with an increase of a histidine C(2)-proton resonance at 8.40 ppm to 8.45 ppm can be seen. This indicates a direct protonation of histidine residues of superoxide dismutase by autoxidizing adrenalin. The protein in the presence of the other catecholamines caused spectra identical with spectrum (c), giving no indication of a further interaction with these catecholamines besides reduction of superoxide dismutase copper.

Nuclear magnetic resonance proved unsuccessful for monitoring autoxidation products of adrenalin directly during the reaction with superoxide dismutase. After 30 minutes of autoxidation a decoupling of the resonances between 3.0 and 3.2 ppm as well as a shift of the methyl resonance to 2.77 ppm can be detected. This latter resonance, however, amounts to less than 10% of the original N-methyl resonance at 2.6 ppm indicating the small extent of autoxidation under these conditions.

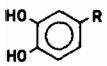
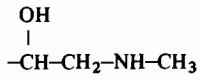
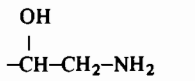
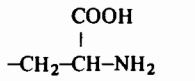
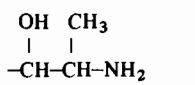
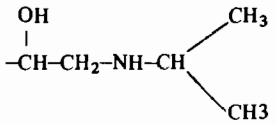
Difference nuclear magnetic resonance spectra of *a*) Cu(II)-superoxide dismutase:Cu(I)-superoxide dismutase, *b*) Cu(I)-superoxide dismutase:superoxide dismutase and adrenalin, and *c*) superoxide dismutase and 3 adrenalin:superoxide dismutase and adrenalin verify that information about oxidation products of adrenalin during this specific interaction is negligible. The N-methyl resonance solely remains detectable under these conditions. All other adrenalin resonances overlap protein resonances and cannot be distinguished.

X-ray Photoelectron Spectrometry

From the observation that borate complexed adrenalin could not prevent the formation of the ternary complex with superoxide dismutase, the involvement of the catecholamine side chain during complexation was deduced [8]. As the amino functional group of the side chains is characteristic for catecholamines, it was interesting to compare the properties of the differently substituted catecholamine nitrogens (Table II). X-ray photoelectron spectrometry is suitable as it allows the determination of binding energies of core electrons, thus giving information about oxidation states or electron densities of particular atoms contained in a compound [29]. The binding energy value of the adrenalin nitrogen 1s electron is unusually low compared to noradrenalin, dihydroxyphenylalanine and the other catecholamines (Table II).

The binding energies do not reflect the extent of alkylation of the different nitrogen atoms. However, as they correlate with the ability of superoxide dismutase to inhibit the autoxidation of adrenalin, noradrenalin and dihydroxyphenylalanine in a way that increasing nitrogen 1s electron binding energies cause less inhibition of the reaction with the protein (Table I), the specific interaction of the side chain nitrogen atom with superoxide dismutase is further supported. This implies that the binding being

TABLE II. 1s Nitrogen Binding Energies of Catecholamines.[a]

Catecholamine 	R	N-1s-Electron Binding Energy eV
Adrenalin		400.3
Noradrenalin		400.9
Dihydroxyphenylalanine		401.0
α-Methyl-noradrenalin		400.9
Epinine	$-CH_2-CH_2-NH-CH_3$	400.8
N-Isopropylnoradrenalin		400.8
Dihydroxyphenylethylamine	$-CH_2-CH_2-NH_2$	400.7
Dihydroxybenzylamine	$-CH_2-NH_2$	401.1

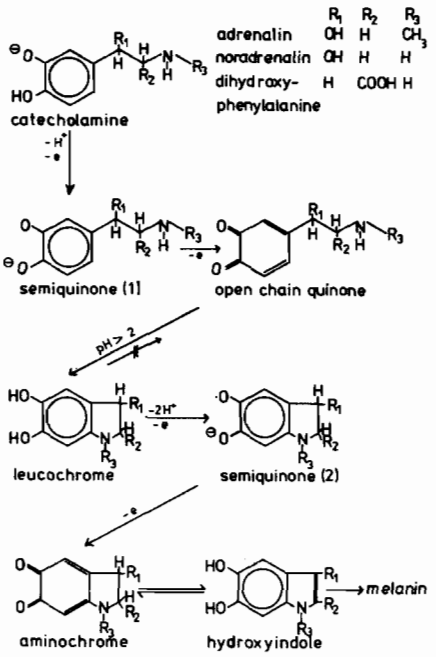
[a] Powdered samples were attached by cellotape to cylindrical aluminum sample holders and binding energies determined at 24 °C. Settings were: X-ray source $Mg_{K\alpha_{1,2}}$ = 1253.6 eV at 10 kV and 100 mA, workfunction 6 eV, sweep width 20 eV, sweep time 20 s, no. of scans 10, no. of channels 200, pressure 3×10^{-4} Pa. At least 3 independent determinations allowed the determination of binding energies to ±0.1 eV.

strongest in the case of adrenalin thus enabling complex formation with the protein. Unfortunately, due to the numerous nitrogens in the superoxide dismutase polypeptide chain, X-ray photoelectron spectrometric measurements of the ternary complex gave a broad unresolved nitrogen 1s signal overlapping the nitrogen 1s signal of adrenalin.

Discussion

The experiments still do not fully explain the specific inhibition of adrenalin autoxidation by superoxide dismutase. However, they are sufficient to establish the formation of a specific ternary complex between the enzyme and oxidizing adrenalin on a molecular basis. To explain this, it is important to consider the autoxidation in more detail. The role of superoxide radicals during the autoxidation of catecholamines remains unclear [30]. These radicals and hydrogen peroxide can be excluded as mediators of the reaction, considering also their high reduction potentials at pH 7 (+1.12 V; +1.35 V [31]) compared to the progressively lower oxidation potentials of oxidizing catecholamines (+0.38 V to +0.01 V [32, 34]). In the presence of transition metal ions, the autoxidation is stimulated concomitantly with the respective ions [8, 13, 33] and proceeds like the uncatalyzed autoxidation in the generally accepted reaction (Scheme 1) [23, 27, 34].

Monitoring autoxidation reactions at wavelengths of aminochromes includes the possibility that melanization is observed [28, 34]. It is believed that autoxidation of noradrenalin and dihydroxyphenylalanine having comparably fast reaction rates until a plateau value is reached within 15–20 minutes, readily convert to melanin products. Adrenalin autoxidation, however, leads to the formation of maximum absorbance within ten minutes of the reaction, corresponding exclusively to adrenochrome formation [28]. In order to explain this phenomenon, the hypothesis is favoured that adrenalin



Scheme 1: Autoxidation of Catecholamines

autoxidation is directed by the reduction potentials of three different couples [34] (determined at ~pH 7):

a)	adrenalin/adrenalin quinone	+ 0.38 V	[32]
b)	leucoadrenochrome/adrenochrome	+ (0.1–0.2) V	[34]
c)	leucoadrenochrome/hydroxyindole adrenochrome/compounds	+ 0.01 V	[34]

Due to the cyclization of adrenalinquinone [17] and the fast oxidation of leucoadrenochrome, adrenochrome accumulates during the stage of steady oxidation. The increasing adrenochrome concentration lowers redox potential b) below that of couple c) which in turn leads to consumption of the accumulated adrenochrome by further oxidation of hydroxyindole compounds to melanin. It is suggested that due to a missing lag phase and continuous conversion of noradrenalin and dihydroxyphenylalanine to melanin products, the reaction steps involved in these autoxidations do not depend on similar potentials. However, it has to be emphasized that this explanation is tentative, based on the fact that methylene blue is reduced only during oxidation of adrenalin at alkaline pH [34].

For this reason and considering the reactivity of borate-inhibited adrenalin [8], it was interesting to examine the electron environments of the catecholamine side chain nitrogen atoms. The low nitrogen 1s electron binding energy value of adrenalin as revealed by X-ray photoelectron spectrometry is indicative of an increased electron density at this atom contrary to those in noradrenalin, dihydroxyphenylalanine and other catecholamines. It is important to note that the extent of alkylation of the nitrogen atoms of the various catecholamines does not alter the binding energies. It may be concluded that both alcoholate and N-alkyl groups affect the electron densities of the nitrogen atoms. Therefore the observation of the low adrenalin nitrogen 1s binding energy becomes significant. This may further be substantiated taking into account the experimental conditions where only solids could be measured. Negative induction effects observeable in solution remained ineffective and might further increase the electron density on the nitrogen atoms. The highest electron density of the adrenalin nitrogen atom supports the idea of complex formation with the protein moiety of superoxide dismutase via the ethanolamine side chain. This suggestion is further substantiated by looking at the reaction mechanism of the autoxidation, which usually leads to indole compounds (Scheme 1) [27, 28]. The rate of cyclization of the open chain quinone to indole compounds is thought to be a rate-limiting step during the oxidation of catecholamines and was shown to vary considerably between different molecules [17]. The fact that electron densities of the nitrogen atoms parallel the cyclization rate constants of different catecholamines may explain the complete inhibition of adrenochrome formation in the presence of superoxide dismutase. As adrenochrome is formed via indole compounds, bonding of adrenalin via its side chain to the enzyme will certainly prevent adrenochrome formation.

Circular dichroism measurements support the hypothesis of ternary complex formation. Monitoring autoxidation of catecholamines by this method revealed significantly new Cotton effects only in the combined presence of superoxide dismutase and adrenalin. The protein conformation was altered in the 250–450 nm region attributable to conformational changes of its copper chromophore. Since reduction of the active site copper of the enzyme is observed, the binding of adrenalin to the protein *via* its side chain is at a site close to the metal centers. Both the protein portion and the metals of superoxide dismutase are essential for the ternary complex formation with adrenalin as may be deduced from the conformational changes of either the Zn_2- or the Cu_2-reconstituted apoprotein. Photochemical destruction of the imidazolate bridged metal centers of superoxide dismutase is sufficient to suppress all conformational changes indicative of a ternary complex between adrenalin and the protein. It appears that besides the involvement of protein-copper in electron transport reactions the structural organization of the bridged metal centers is a prerequisite for the catalytic action of superoxide dismutase.

Using proton resonance spectrometry this conclusion can definitely be supported. As in the circular dichroism studies a partial reduction of the protein-

copper can be deduced comparing the difference spectra. The distinct difference spectrum of dithionite-reduced and the oxidized protein can also be obtained at much less intensity when the one of adrenalin-reduced is subtracted from the dithionite-reduced. This is taken as evidence for incomplete reduction in these experiments. This latter difference spectrum also reveals that except the N-methyl proton resonance no proton resonances of adrenalin and/or its oxidation products are observeable under the incubation conditions.

However, the method allows the demonstration of a proton transfer from oxidizing adrenalin to superoxide dismutase. The reaction (Scheme 1) reveals that during conversion of leucoadrenochrome to the second semiquinone liberation of protons takes place. The resonance increase of histidine protons (Fig. 4 c) prooves that liberated protons directly bind to these residues. As His 41 is buried in the polypeptide chain and thus unlikely to participate in a chemical reaction, and His 19 resonances are assigned at 7.45 ppm [14, 15], the protonation of the remaining six active site histidines may be concluded. The small chemical shift of the C(2)-histidine resonance at 8.40 ppm to 8.45 ppm may also be an indication of the adrenalin induced conformational change of the superoxide dismutase active site. However, a closer assignment of histidine resonances is required and it remains to be demonstrated that the proton transfering imidazolate bridge between copper and zinc is involved in the catalytic mechanism, as already proposed [35]. Possibly more information about the catalytic mechanism of the reaction of superoxide dismutase will be obtained with active site models of superoxide dismutase [36].

From the experiments we draw the conclusion that the specific inhibition in the presence of superoxide dismutase of the autoxidation of adrenalin is caused by binding to the protein via the ethanolamine side chain. The oxygen–adrenalin–protein adduct prevents the cyclization of adrenalinquinone to leucoadrenochrome and thus considerably affects the redox-equilibria of adrenalin and its oxidation products. Adrenochrome formation is excluded in the presence of superoxide dismutase, although oxidation of adrenalin is still observeable. The formation of the postulated ternary complex between superoxide dismutase and oxidizing adrenalin may account for the suppression of the formation of *e.g.* superoxide radicals during adrenalin semiquinone formation. A reaction of superoxide dismutase with such transient radicals before superoxide is dissociated would facilitate the explanation of the occurrence of this enzyme in anaerobes [37].

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