Redox Behaviour and Stability of Active Centre Analogues of Cu_2Zn_2-Superoxide Dismutase

MARGOT LINSS and ULRICH WESER

Anorganische Biochemie, Physiologisch-chemisches Institut der Universität Tübingen, Hoppe-Seyler-Strasse 1, D-7400 Tübingen, F.R.G.

(Received May 11, 1987)

Abstract

Active site analogues of Cu_2Zn_2-superoxide dismutase were devised and successfully employed. The chelates of the di-Schiff-bases pyridine-2-aldehyde, imidazole-2-aldehyde and 1,4-diaminobutane, respectively, were shown to mimick both structure and function of the copper binding centre of the native enzyme. Electronic absorption, magnetic properties and redox potential were of intriguing similarity. The redox potential of the $CuL_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$ was at 0.21 V and was very close to the 0.27 V measured for Cu_2Zn_2-superoxide dismutase. The stability constant of $CuL_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$ was $\log K = 16.1$. $CuL_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$ was almost one order of magnitude more stable ($\log K = 17.1$) compared to that of Cu(II)–serum albumin ($\log K = 16.2$).

Introduction

Cu_2Zn_2-superoxide dismutase is a well characterized example of the type II copper proteins [1, 2]. During the catalysis of superoxide dismutation the coordinated copper remains in the active centre and changes its redox state from Cu(II) into Cu(I) [3]. Initially the Cu(II) is found in a distorted square planar arrangement which is converted into a tetrahedral structure. The protein backbone is flexible enough to follow these conformational changes quite effectively. By way of contrast there are low M_r Cu–chelates where Cu(II) is found in an acetate or biuret structure [4, 5]. These Cu-complexes exert idential rate constants using pulse radiolytically generated superoxide. Attributable to their low thermodynamic stability the transiently formed Cu(I) species are poor substitutes for the active site of the native enzyme. Thus, flexible mononuclear Cu-complexes were devised and prepared employing the di-Schiff-base of pyridine-2-aldehyde and 1,4-diaminobutane [6]. Replacement of pyridine-2-aldehyde by imidazole-2-aldehyde was expected to improve the stability. Of special interest was to compare the stability of these two Cu(II)–chelates and Cu(II)–serum albumin. Circular dichroic measurements were employed for this task. Provided these flexible Cu(I)/Cu(II)–chelates are genuine active site analogues of Cu_2Zn_2-superoxide dismutase the redox potentials should be similar. Cyclic voltammetry was used to shed some light on this question.

Experimental

Chemicals

Pyridine-2-aldehyde, imidazole-2-aldehyde and 1,4-diaminobutane were from Ega Chemie, Steinheim; bovine Cu_2Zn_2-superoxide dismutase was isolated from red blood cells [7]. {[*N,N'*-Bis(2-pyridylmethylene)-1,4-butanediamine]-(*N,N',N'',N'''*)}-Cu(II)diperchlorate ($CuL_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$) was prepared as in ref. 6. {[1.8-Di(2-imidazoyl)-2.7-diazooctadien-1.7]-(*N,N',N'',N'''*)}Cu(II)diperchlorate ($CuL_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$) was synthesized as in ref. 8. Elemental analysis of C, H, N and Cu varied between ±2% from the theoretical values. Xanthine, xanthine oxidase and nitrotetrazolium blue were from Serva, Heidelberg.

Spectrometry

Circular dichroism was recorded on a JASCO J 20A spectropolarimeter. Electron paramagnetic resonance was measured at 100 K on a Varian E 109 EPR spectrometer. Copper was determined employing a Perkin-Elmer 400 atomic absorption spectrometer furnished with a HG 76B graphite furnace. Elemental analysis of C, H and N were preformed on a Perkin-Elmer Elemental Analyzer 240B.

Cyclic Voltammetry

In relation to the potentiometric titration of Cu_2Zn_2-superoxide dismutase [9], cyclic voltammetry was carried out using a potentiostat LB81 equipped with a scanner VSG72, Bank Elektronik, Göttingen. A three-electrode assembly was employed consisting of a glassy carbon disk electrode, a

0020-1693/87/$3.50

Pt auxiliary electrode and saturated calomel as a reference electrode. Attributable to the half wave potentials of the employed Cu-complexes all measurements were carried out in aqueous 80 mM KCl under nitrogen. The Cu-concentration was 2.3 mM. Cycles were run from −0.4 to 0.6 V at a scan rate of 0.03, 0.05, 0.1 and 0.2 V/s on a Honeywell 530 XY recorder.

Superoxide Dismutase

Superoxide dismutase was assayed in comparing the reduction of nitro-blue tetrazolium in the presence and absence of Cu-complexes. As in ref. 6 xanthine/xanthine oxidase served as a convenient superoxide source.

Results and Discussion

Copper Coordination

It is well known that dicarbonylic compounds including ketones or aldehydes react with a primary amine attached to a nitrogen containing heterocycle. The resulting tetradentate ligand provided 4 unsaturated nitrogen atoms capable to coordinate both Cu(II) and Cu(I). The Cu(II)-coordinated pyridine-2-aldehyde or imidazole-2-aldehyde and 1,4-diaminobutane yielded di-Schiff-bases most suitable to mimick the active centre of Cu_2Zn_2-superoxide dismutase. As in the case of the native enzyme the nitrogen atoms could follow the conformational change from the distorted square planar arrangement of Cu(II) to the tetrahedrally surrounded Cu(I) (Fig. 1). The aliphatic C_4-unit originating from 1,4-diaminobutane ascertains the flexible nature or the Cu-chelator. Either copper chelate was soluble in aqueous systems where they turned out to be efficient catalysts to accelerate superoxide dismutase.

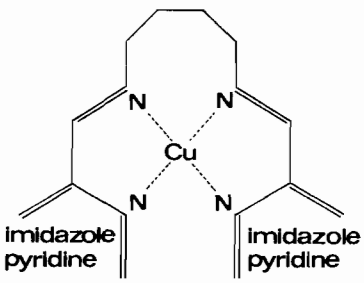

Fig. 1. Schematic structure of flexible active site analogues of Cu_2Zn_2-superoxide dismutase.

Superoxide Dismutase Activity

The two active site analogues displayed a considerable superoxide dismutase mimicking activity. It was 20–60 times higher compared to that of low M_r-Cu–chelates of the biuret or acetate type reported earlier [10, 11] (Table I). Obviously, the flexible nature of the di-Schiff-base copper chelates improved the enzymic activity. Unlike the native enzyme where four imidazolate allow the convenient electron dislocation the diminished activity of the present active site analogues may be assigned to the linking butylic moiety which is unable to facilitate electron dislocation.

TABLE I. Catalytic Activity of Cu_2Zn_2-superoxide Dismutase and Cu–Chelates of the Di-Schiff Bases Employing Pyridine-2-aldehyde and Imidazole-2-aldehyde[a]

Copper chelate	μM Cu(II) required for 50% inhibition of formazane generation
Cu_2Zn_2-superoxide dismutase	0.04
$CuL_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$	1.4
$CuL_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$	4.0
Cu(acethylsalicylate)$_2$ [10]	23.0
Cu(lysine)$_2$ [11]	86.0

[a]Assay conditions: 0.5 ml reaction volume, 50 μM xanthine, 0.18 μM xanthine oxidase, 0.62 mM nitro blue tetrazolium, 0.2%(*w*/*v*) gelatine, 20 mM Hepes buffer, pH 7.4, 150 mM NaCl. A_{540} was registered at 23 °C.

Electronic Absorption and Magnetic Properties

The weak absorption between 680 and 710 nm is typical for d–d transitions of Cu(II) in a weak tetragonal field. Unlike the native enzyme a redshift by 10–30 nm is seen when the di-Schiff-bases are employed. Cu_2Zn_2-superoxide dismutase has its maximum at 680 nm ($\epsilon_{Cu} = 150\ M^{-1}\ cm^{-1}$), $CuL_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$ at 710 nm ($\epsilon_{Cu} = 101\ M^{-1}\ cm^{-1}$) and $CuL_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$ at 690 nm ($\epsilon_{Cu} = 35\ M^{-1}\ cm^{-1}$).

The electronic paramagnetic resonance spectra are of striking similarity to the native enzyme (Table II, Fig. 2) [12, 13]. $g_\parallel/A_\parallel$ is an empirical factor to characterize the degree of tetrahedral distortion of a tetragonally arranged complex [14]. Between 105 and 135 cm square planar arrangements are detectable. A progressive rise of up to 250 is indicative for tetrahedral distortion. In fact the 134 cm of the di-Schiff-base–Cu-chelates are at the borderline of such a tetrahedral arrangement and agree fairly well with the EPR data observed with native Cu_2Zn_2SOD and Cu_2E_2SOD.

Cyclic Voltammetry

Potentiometric titrations of bovine Cu_2Zn_2-superoxide dismutase and evaluation of the oxidation–reduction potential revealed a value of +0.27 V [9]. Cyclic voltammetry of the employed copper chelates should additionally demonstrate the reversibility of the oxidative process. Much to our surprise, the redox potential of the employed $CuL_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$ was almost the same and was determined at 0.21 V. Furthermore, the redox cycle

TABLE II. Comparison of EPR Data of Active Site Analogues and Cu_2Zn_2-superoxide Dismutase

Copper chelate	$g_\perp$	$g_\parallel$	$A_\parallel$		$g_\parallel/A_\parallel$
			(G)	$\times 10^{-4}$ (cm^{-1})	(cm)
Cu_2Zn_2-superoxide dismutase [12]	2.087	2.268	134	142	160
Cu_2E_2-superoxide dismutase [13]	2.067	2.268	146	155	146
$CuL_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$	2.047	2.234	160	166	135
$CuL_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$	2.040	2.226	160	166	134

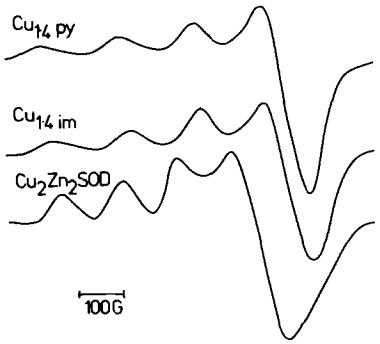

Fig. 2. Electron paramagnetic resonance spectra of Cu_2Zn_2-superoxide dismutase, $Cu_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$ and $Cu_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$. The instrumental settings were: microwave power 20 mW; frequency 9.24 GHz; modulation amplitude 10 G; temperature 100 K.

proved to be reversible (Fig. 3). No such reversibility was seen when $CuL_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$ was used. Therefore, the precise assignment of this redox potential was not made. Whether or not this phenomenon can be attributed to either inhibited electron transfer onto the electrode or to chemical modification of the Cu-complex awaits further investigations.

Stability Constants

The competitive Cu(II) binding capacity of the di-Schiff-bases and serum albumin was compared to determine the stability constant of the employed $CuL_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$ and $CuL_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$. Fortunately, no chiroptic properties of the

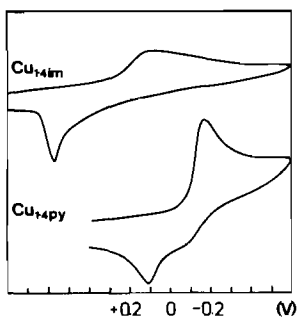

Fig. 3. Cyclic voltammetry of $Cu_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$ and $Cu_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$. The scan rate was 0.1 V/s, the copper concentration was 2.4 mM.

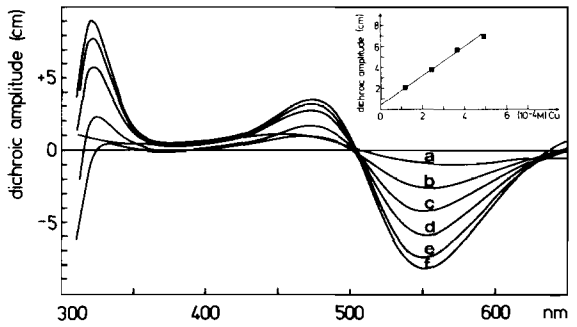

Fig. 4. Circular dichroism of bovine serum albumin titrated with rising concentrations of $Cu(ClO_4)_2$ in mM: (a) 0; (b) 0.13; (c) 0.25; (d) 0.37; (e) 0.5; (f) 0.61. The serum albumin concentration was 0.6 mM. Inset: Calibration curve of dichroic amplitude at 550 nm *versus* Cu(II) concentration.

active site analogues are noticed in the 550 nm region (Fig. 4). The characteristic negative Cotton band at 550 nm of Cu–serum albumin was used to follow the displacement from the low M_r chelates. Human and bovine serum albumin they both have distinct Cu(II) binding sites on the N-terminal end [15]. The stability constant of these binding sites are several orders of magnitude higher compared to the many other unspecific binding sites in the protein. Thus, Cu(II)L is competing with the former Cu(II) binding site of serum albumin only.

$$\text{Cu(II)L} \rightleftharpoons \text{Cu(II)} + \text{L} \quad (1)$$

$$\text{Cu(II)} + \text{BSA} \rightleftharpoons \text{Cu(II)BSA} \quad (2)$$

$$\text{Cu(II)L} + \text{BSA} \rightleftharpoons \text{Cu(II)BSA} + \text{L} \quad (3)$$

A stability constant relative to that of Cu(II)BSA can be calculated (Table III).

$$\beta_{rel} = \frac{K_{Cu(II)BSA}}{K_{Cu(II)L}} = \frac{[Cu(II)BSA]\,[L]}{[BSA]\,[Cu(II)L]} \quad (4)$$

([L] = Cu(II)BSA, Cu(II)L = Cu(II) – [Cu(II)BSA], [BSA] = BSA – [Cu(II)BSA])

From eqn. (4) the absolute value of the stability constants can also be obtained. Assuming log K = 16.2 for [Cu(II)BSA] [15] the corresponding values

TABLE III. Determination of Stability Constants Relative to CuBSA[a]

Cu(II) (mM)	a (cm)	[Cu(II)BSA] ≙ [L] (mM)	[Cu(II)L] (mM)	[BSA] (mM)		β_{rel}
$CuL_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$						
0.262	3.1	0.190	0.072	0.410		1.22
0.393	3.5	0.252	0.141	0.348		1.29
0.524	4.2	0.280	0.244	0.320		1.00
					mean	1.17 ± 0.16
$CuL_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$						
0.240	2.5	0.150	0.090	0.450		0.55
0.360	3.2	0.195	0.165	0.405		0.57
0.480	3.5	0.219	0.261	0.381		0.48
0.600	3.6	0.232	0.368	0.368		0.40
					mean	0.50 ± 0.07

[a]The bovine serum albumin concentration was 0.6 mM. All titrations were carried out in 20 mM Hepes buffer at pH 7.4. a is the dichroic amplitude.

are $\log K = 16.2$ for $CuL_{1,4py}(ClO_4)_2 \cdot 0.5H_2O$ and $\log K = 17.1$ for $CuL_{1,4im}(ClO_4)_2 \cdot 0.5H_2O$. Either Cu(II) complex is stable enough to have a fair chance to survive, at least in part, in biological systems.

Conclusion

A genuine active site analogue of Cu_2Zn_2-superoxide dismutase should permit to coordinate both Cu(II) and Cu(I). During the catalytic process the square planar arrangement of superoxide dismutase around the Cu(II) is abandoned and Cu(I) is tetrahedrally coordinated. In the native enzyme the first shell atoms are four unsaturated nitrogens originating from histidine moieties. Four unsaturated nitrogen atoms are provided in essentially the same manner using the di-Schiff-bases of pyridine-2-aldehyde, imidazole-2-aldehyde and 1,4-diaminobutane, respectively. The butyl moiety ascertains the flexibility of the chelator in a way similar to the protein backbone in the native enzyme. All physicochemical properties turned out to be quite similar to those of the native protein. Even the thermodynamic stability of the Cu-complexes is close to that of the major serum Cu(II)–protein. The biochemical consequences in many a reaction where $\cdot O_2^-$ is a deleterious or beneficial intermediate will be awaited with great interest.

Acknowledgements

This study was aided by DFG-grants We 401/17/18 and in part by the Fonds der Chemischen Industrie. Further thanks go to Helga Heinemann and Peter Urban for expert technical help.

References

1 A. Gärtner and U. Weser, in F. L. Boschke (ed.), 'Topics in Current Chemistry', Vol. 132, Springer-Verlag, Berlin, 1986, pp. 1–61.
2 B. Reinhammar and B. C. Malmström, in T. G. Spiro (ed.), 'Copper Proteins', Wiley, New York, 1981, pp. 109–149.
3 G. Rotilio, R. C. Bray and E. M. Fielden, *Biochim. Biophys. Acta, 268,* 605 (1972).
4 U. Weser, K.-H. Sellinger, E. Lengfelder, W. Werner and J. Strähle, *Biochim. Biophys. Acta, 631,* 232 (1980).
5 R. Brigelius, H.-J. Hartmann, W. Bors, M. Saran, E. Lengfelder and U. Weser, *Z. Physiol. Chem., 356,* 739 (1975).
6 M. Linss and U. Weser, *Inorg. Chim. Acta, 125,* 117 (1986).
7 A. Gärtner, H.-J. Hartmann and U. Weser, *Biochem. J., 221,* 549 (1984).
8 M. Linss, *Dissertation,* Tübingen, 1986.
9 G. D. Lawrence and D. T. Sawyer, *Biochemistry, 18,* 3045 (1979).
10 U. Weser, C. Richter, A. Wendel and M. Younes, *Bioinorg. Chem., 8,* 201 (1978).
11 M. Younes and U. Weser, *FEBS Lett., 61,* 209 (1976).
12 G. Rotilio, A. Finazzi-Agro, L. Calabrese, F. Bossa, P. Guerrieri and B. Mondovi, *Biochemistry, 10,* 616 (1971).
13 K. M. Beem, D. C. Richardson and K. V. Rajagopalan, *Biochemistry, 16,* 1930 (1977).
14 U. Sakaguchi and A. W. Addison, *J. Chem. Soc., Dalton Trans.,* 600 (1979).
15 S. J. Lau and B. Sarkar, *J. Biol. Chem., 246,* 5938 (1971).