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Coagulation of Nickel-modified Hemoglobin

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Abstract — Kinetics of coagulation of hemoglobin, in particular, of bovine oxyhemoglobin, in the presence of a Ni(II) salt was measured for the first time. The initial coagulation rate is several times lower than the initial rates of hemoglobin coagulation in the presence of Hg(II), Cd(II), and Zn(II) salts. In hemoglobin at neutral pHs, there is a center of preferential binding with nickel ions (presumably, reactive thiol groups of cysteine), but increasing temperature and pH disturb the selectivity of binding with this center. Hydroxide anions essentially slow down the coagulation rate, which is attributable to their competition for nickel cations.

The wide use of nickel in engineering gave rise to a great number of publications dedicated to the chemical, physical, and toxicological characteristics of nickel compounds. In particular, the chemistry of nickel coordination compounds is adequately reflected in the review [1]. Nickel complexes with proteins have been studied to a much lesser extent (see, for example [2]). Among recent reviews concerning nickel complexes with amino acids, peptides, and proteins, the review [3] is worth mentioning. At the same time, we failed to find publications dedicated to reactions of nickel salts with hemoglobin. By choosing nickel as the next metal in our research on the influence of metal ions on the kinetics of hemoglobin aggregation (coagulation), we thus undertook the first study of Ni(II) interaction with this protein.

In this work we made use of turbidimetry to study the kinetics of coagulation of bovine oxyhemoglobin (HbO_2) in a Tris–acetate buffer in the presence of Ni(II) chloride. In particular, we dwelt on various concentration dependences and temperature and pH effects.

Numerous studies of Ni(II) complexes established that the preferred configuration of the metal is octahedral, though sometimes square-planar and distorted configurations are realized [1]. Thus it would be expected that, occupying an intermediate place between typical soft (mercury and cadmium) and hard (light alkali metals) metals, nickel is roughly equally available for bonding with hard (oxygen) and soft (sulfur) centers. In particular, the ability of the cysteine SH group to bind effectively the Ni(II) ion has been reported [4, 5].

Our experiments showed that ~10^{-4} M HbO_2 rather effectively coagulates in the presence of ≥2 mol of nickel ions per 1 mol of HbO_2 tetramer, though the initial rate of coagulation of nickel-modified HbO_2 is several times lower than the initial rate of HbO_2 coagulation in the presence of the same concentrations of Hg(II), Cd(II), and Zn(II) ions. Despite the quantitative difference, there is a good reason to think that the mechanism of hemoglobin coagulation in the presence of all mentioned metals taken in a restricted concentration is the same. Since the selectivity of binding of Hg(II) ions with the cysteine sulfur atom is rather high, we can suggest that other metal ions, nickel inclusive, too, primarily bind with sulfur, which causes loss of protein aggregative stability.

It is necessary to note that it is incorrect to compare only the bond strength of the nickel ion with sulfur and other simple binding centers (aromatic and aliphatic nitrogen, carboxylate oxygen, etc.), as we suppose that free valent orbitals of the nickel ion bound with sulfur can interact with adjacent protein binding centers, and, as a result, the polydentate binding with the metal can increase the strength of binding of the nickel ion with sulfur by several orders of magnitude.

Figure 1 shows a typical series of kinetic curves of HbO_2 coagulation at 30°C in a 0.05 M Tris–AcOH buffer (pH 7.2) for an HbO_2 concentration of 1×10^{-4} M (per tetramer) and various concentrations of nickel chloride.

All the curves in Fig. 1 were approximated by the second-order equation (1) we deduced earlier for turbidimetric experiments [6].

$$D = (D_{max})^2 k(t + t')/[1 + D_{max} k(t + t')]. \quad (1)$$

Here D is the current apparent optical density of the turbid medium; D_{max} is the limiting D value at

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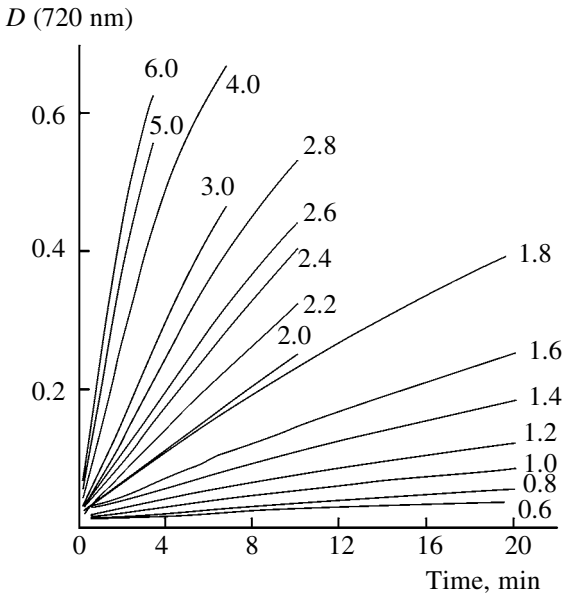

Fig. 1. Kinetic curves of coagulation of nickel-modified HbO_2 at various concentrations of $NiCl_2$ [molar concentrations of $NiCl_2$ ($\times 10^4$) are indicated on the curves] and constant concentration of HbO_2 (1×10^{-4} M, per tetramer) in 0.05 M Tris–Ac (pH 7.2) at 30°C.

maximum coagulation; k is the second-order rate constant, min^{-1}; t is the real time, min; t' is the time correction for initial deviations of the kinetic curves from ideal second-order curves. By definition, $(D_{max})^2 \cdot k$ (min^{-1}) is the initial coagulation rate, which is a conventional value to a certain extent, as it does not include the concentration of a substance (specifically, coagulated protein) but only characterizes its relative change.

The dependence of the initial coagulation rate $v_0 = (D_{max})^2 k$ on the concentration of nickel chloride is given in Fig. 2a. The solid line in a plot drawn through experimental points and the broken line is the exponential fit by Eq. (2).

$$v_0 = (D_{max})^2 k = (v_0)_0 + ax^n. \quad (2)$$

Here $(v_0)_0$, a, and n are experimental parameters.

The semilog anamorphosis (Fig. 2b) of the experimental curve displays an ill-defined inflection at a concentration ~2 M.

Similar series of experiments were performed with HbO_2 concentrations of 0.75×10^{-4} and 0.5×10^{-4} M. The resulting data were also fit by Eq. (2), and corresponding semilog plots were constructed. The plot of log v_0 vs. $[NiCl_2]$ for the HbO_2 concentration of 0.75×10^{-4} M, too, has an inflection, whereas in that

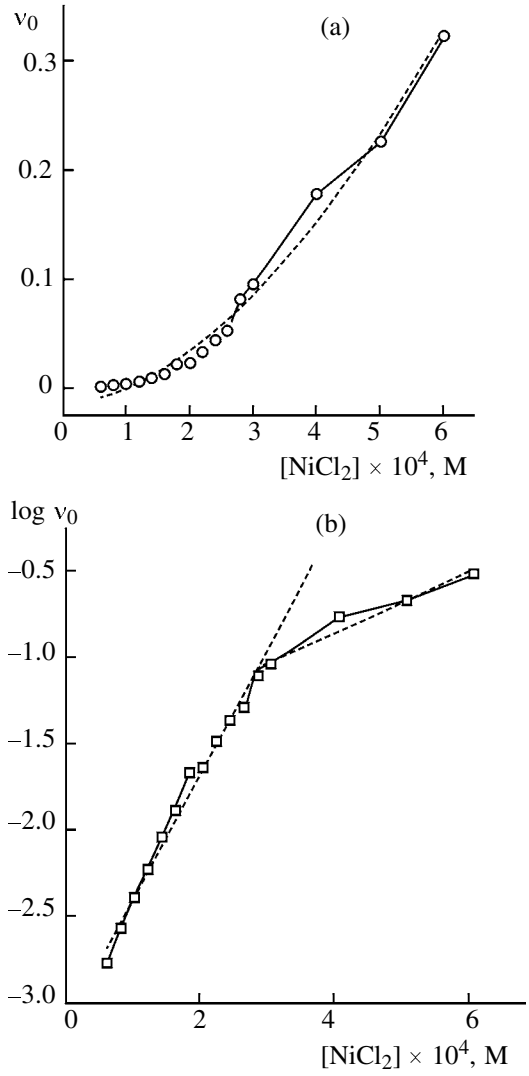

Fig. 2. Dependence of the initial rate (v_0) of coagulation of nickel-modified HbO_2 l on the concentration of $NiCl_2$ by the data of Fig. 1. (a) (Solid line) experimental curve and (broken line) curve calculated by equation (2). (b) Semilog anamorphosis of the experimental curve [(broken line) separate rectification of the initial (4 points) and final (4 points) portions of the experimental curve].

for the HbO_2 concentration of 0.5×10^{-4} M no inflection is defined. All calculated parameters for the three HbO_2 concentrations are given in Table 1.

The data in Table 1 reveal the following regularities. The exponent n in Eq. (2) which takes relatively little account of low initial coagulation rates is the same for all the three protein concentrations and close to 2. At the same time, as seen from the semilog plots, at two relatively high protein concentrations after the inflection point log v_0 begins to much slower increase

Table 1. Comparative characteristics of HbO_2 coagulation at its various concentrations

$[HbO_2] \times 10^4$, M	Parameters of Eq. (2)			Slopes of semilog curves		Approximate position of inflection in the semilog curve [Ni]/[Hb]
	v_0	a	n	initial branch	final branch	
0.5	–0.0002 ± 0.0031	0.0045 ± 0.0018	1.9641 ± 0.2707	0.4261 ± 0.0332	–	–
0.75	–0.0087 ± 0.0117	0.0069 ± 0.0049	1.6989 ± 0.3653	0.6808 ± 0.0530	0.2279 ± 0.0267	~3.2
1.00	–0.0341 ± 0.0355	0.0396 ± 0.0186	2.1022 ± 0.3261	1.2384 ± 0.1088	0.3179 ± 0.0664	~2.2

Table 2. Slopes of the first and second branches of the curves in Fig. 3

Temperature, °C	Slope of the first branch	Slope of the second branch	Approximate position of inflection in the semilog curve ([Ni]/[Hb])
20	1.462 ± 0.156	0.422 ± 0.084	~2.8
25	1.199 ± 0.045	0.370 ± 0.039	~2.4
30	1.238 ± 0.109	0.318 ± 0.066	~2.2
35	1.138 ± 0.175	0.190 ± 0.041	~1.9
40	1.121 ± 0.170	0.556 ± 0.116	~2.2

with nickel chloride concentration. As the protein concentration decreases, the slopes of the two branches of the curves tend to get closer together, and at the HbO_2 concentration of 0.5×10^{-4} M the difference between the slopes of the initial and final branches is no longer valid.

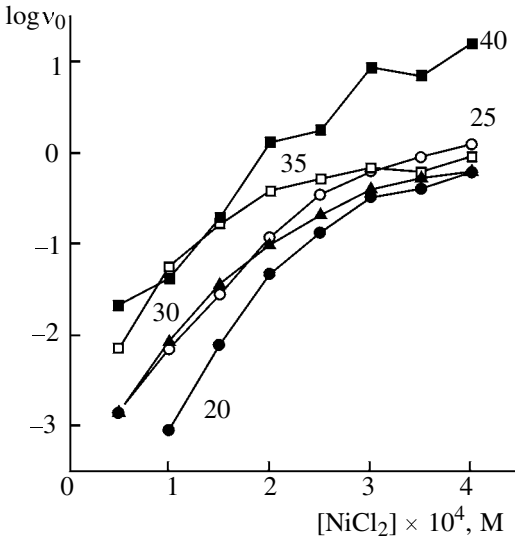

Fig. 3. Effect of $NiCl_2$ concentration on the initial rate of HbO_2 coagulation at various temperatures (°C, given on curves). Concentration of HbO_2 1×10^{-4} M, 0.05 M tris–AcOH, pH 7.2.

Varying the temperature gave the following results. All the plots of $\log v_0$ vs. nickel chloride concentration for a series of temperatures (Fig. 3) have a better or worse defined inflection at the $NiCl_2$ concentrations from 2×10^{-4} to 2.5×10^{-4} M. This corresponds to the Ni/HbO_2 ratio 2–2.5. The best defined inflection is observed at 25 and 30°C. Table 2 lists the slopes of the first and second branches of the curves at all the temperatures studied.

The rather intricate nature of the temperature effect can be accounted for by different temperature responses of the factors determining the initial rate of protein coagulation: the degree of exposition of binding centers and the corresponding distribution of nickel ions over thiol and nonthiol centers, the equilibrium between dimeric, tetrameric, and higher oligomeric forms of the protein; the equilibrium between the high-spin and low-spin states of nickel in complexes with binding centers in the protein [1], etc. Nevertheless, certain regularities in Fig. 3 are obvious.

(1) At low concentrations of nickel ions, the temperature rise from 20 to 35–40°C increases the initial rate of HbO_2 coagulation by almost two orders of magnitude. Since the nickel ion is rather strongly bound primarily with the sulfur atom of one of reactive thiol groups, temperature can only affect the protein state and protein–protein interaction. Here we can arbitrarily argue that the temperature effect is Arrhenius in nature, assuming a very high activation energy of protein aggregation.

(2) In the case of a fourfold molar excess of nickel ions, when almost all reactive thiol groups are bound with nickel, and remaining nickel ions interact with nonthiol centers, the temperature rise from 20 to 35°C scarcely affects the initial rate of protein coagulation. Most likely, in this case, increasing temperature shifts to the left equilibrium (3) between tetrameric HbO_2

molecules and linear oligomers [7], which precedes bulk protein aggregation.

$$n/2(\alpha\beta)_2 \rightleftarrows (\alpha\beta)_n. \quad (3)$$

Noteworthy is a rather fast increase in v_0 at 40°C at the $NiCl_2$ concentration higher than 2×10^{-4} M. Apparently, the temperature rise from 35 to 40°C jumpwise loosens the protein conformation to expose new centers, probably, unreactive thiol group of cysteines β112 and α104 and new histidine groups [8] whose binding with nickel enhances protein aggregation.

The state of proteins, including their aggregation, is strongly pH-dependent [9, 10]. However, it can hardly be said that the mechanism of the pH effect on protein aggregation is quite clear [11]. On the one hand, it is widely known that the aggregation stability of proteins is the lowest in the isoelectric point, when intermolecular repulsion is minimal. However, on the other hand, if under close-to-neutral isoelectric conditions a protein has a rather high aggregative stability, then deviation of pH from neutral values to one or the other sides denatures the protein, thereby rendering it aggregatively unstable. In particular, there is some evidence showing that the minimum of thermal denaturation of hemoglobin, as determined by its coagulation, lies in the region of neutral pHs [12].

We have studied the effect of pH on HbO_2 coagulation within the limits of the buffer capacity of Tris (pH from 7.0 to 8.8). Figure 4 presents two plots of log v_0 vs. pH for HbO_2 in the presence of two- and fourfold molar excesses of nickel chloride. In both cases, the initial coagulation rate is maximal at pH 7.5–7.6, i.e. in the pH region where HbO_2 is conformationally quite stable, provided no other denaturing factors are operative. Therefore, the patterns of the curves in Fig. 4 are most probably determined by the effect of the medium on nickel ions and on conditions of their interaction with the protein. In the case of a 2:1 Ni–HbO_2 complex, which represents a simpler model, the coagulation retardation is the most pronounced (almost by an order of magnitude) when pH increases from 7.5 to 8.8. This result was not unexpected for us by the following reasons.

Earlier [13–15] we assumed, based on experiments with mercury, that a metal ion, by binding with a reactive thiol group, reduces protein aggregative activity owing to coordination interaction with carboxyl of one of adjacent aspartic acid residues. Evidence for this assumption was obtained from the observation of coagulation retardation in the presence of a broad range of nucleophilic reagents that competed with the carboxy group of the protein for the

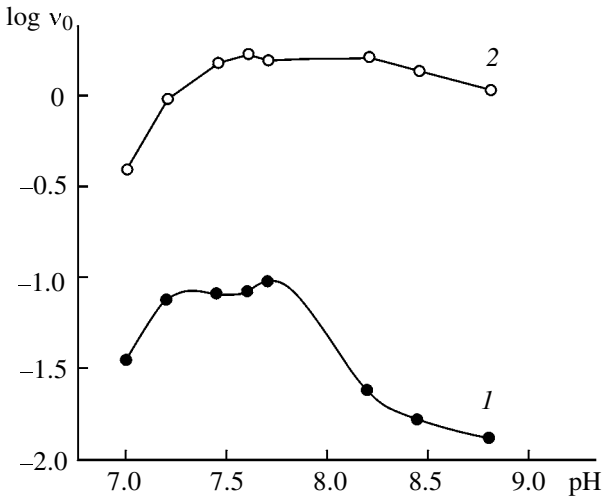

Fig. 4. Effect of pH on the logarithm of the initial rate of HbO_2 coagulation by (*1*) 2 and (*2*) 4 mol of $NiCl_2$.

mercaptide mercury. The hydroxide anion cannot be an exception. However, in contrast to Hg(II), the Ni(II) ion at the sulfur atom under alkaline conditions can not only bind the OH^- ligand, but also leave the sulfur atom together with this ligand, which, too, will entail coagulation retardation. This conclusion is not inconsistent with a much weaker coagulation retardation in the alkaline pH range in the case of a 4:1 Ni–HbO_2 complex. In this case, the weakened binding with sulfur is compensated for by interaction of the metal ion with other centers, which may be differently affected by pH.

The reason for the coagulation retardation at pHs below ~7.5 may be quite different. It is known that decreasing pH of the medium weakens α^1–β^1 dimer–dimer contacts in ligated hemoglobin [16]. Therefore, acidification of the medium, higher linear hemoglobin oligomers that precede coagulate, too, are increasingly destabilized because of the weakened bonding between the dimers forming the linear oligomeric chain.

It should also be noted that in the alkaline pH region beginning already with pH ~7.5, the plots of log v_0 vs. nickel salt concentration have no noticeable inflections, and the slopes of these curves are of the order of 1.0–1.2, i.e. they are close to the slope of the first branch of the curves at fairly low pHs. This result implies that the competition of hydroxide ions for nickel ions substantially levels the affinity of binding protein centers to nickel, and, at relatively high pHs, there is no selective bonding with any protein center.

As an illustration, Fig. 5 shows three plots of log v_0 vs. nickel chloride concentration at various pHs. These curves, unlike those in Fig. 4, obtained with

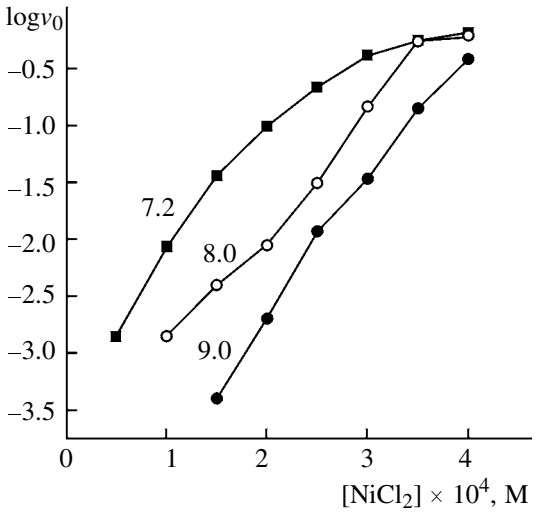

Fig. 5. Plots of the logarithms of the initial rate of HbO_2 coagulation vs. $NiCl_2$ concentration at various pHs (given on curves). Concentration of HbO_2 1×10^{-4} M, 0.05 M Tris–Ac, pH 7.2, 30°C.

different HbO_2 solutions. Therefore, rigorous quantitative comparison of separate curves in Fig. 5 is not quite correct. Nevertheless, common semiquantitative regularities are fulfilled. This set of curves resembles the set of curves obtained under similar conditions with zinc [17]. Except for quantitative differences (on the average, coagulation with zinc is several times faster), the latter curves have inflections at zinc chloride concentrations of $(2–3) \times 10^{-4}$ M in the entire pH range, whereas the curves at pH 8–9 in Fig. 5 are practically linear. In both cases, the rates of coagulation get closer together at increased salt concentrations. Presumably, the main difference between the effects of the metals consists in a more selective binding of zinc ions with cysteine sulfur, which results, on the one hand, in a higher initial rate of coagulation with zinc and, on the other, in the appearance of inflections in the curves even in an alkaline medium. We suppose that the reason for the high selectivity of zinc binding with thiol sulfur is not the strength of the sulfur–metal bond; rather it is a more effective interaction between the sulfur-bound tetrahedral zinc with adjacent electrophilic centers (presumably, aspartic carboxyl) as compared to the nickel ion which is scarcely prone to tetrahedral coordination.

EXPERIMENTAL

Freeze-dried bovine methemoglobin (Serva), pure grade tris(hydroxymethyl)aminomethane recrystallized from alcohol (Tris, Olaine Plant of Chemical Reagents), and chemically pure grade $NiCl_2 \cdot 6H_2O$ (Reakhim) were used.

The procedures of preparation and purification of oxyhemoglobin, as well as turbidimetric kinetic studies (λ 720 nm) have been described in [6, 15].

Data processing was carried out using the mathematical apparatus of an Origin-41 graphic processor.

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