A C T A U N I V E R S I T A T I S L O D Z I E N S I S

FOLIA BIOCHIMICA ET BIOPHYSICA 1, 1981

Wirgiliusz Duda, Zbigniew Hrabec, Wanda Leyko

EFFECT OF 2,3-DPG ON THE STABILITY OF ISOLATED APO-α AND APO-β CHAINS OF BOVINE HEMOGLOBIN

The aim of this study was to investigate whether 2,3-DPG (in a ratio of 2 molecules DPG per 1 molecule Hb-tetramer) exerts an analogous effect on bovine globin chains as ATP. It was also intended to check whether the fractions of free and loosely bound phosphates influence the oxygen affinity of bovine hemoglobin.

MATERIAL AND METHODS

Hemoglobin and globin, α- and β-chains of bovine globin were isolated according to the methods described elsewhere [3].

S p e c t r o p h o t o m e t r i c d e t e r m i n a t i o n o f o x y g e n d i s s o c i a t i o n c u r v e s o f b o v i n e h e m o g l o b i n. Determination of oxygen dissociation curves was performed for:

1. Bovine hemoglobin containing free, loosely- and firmly bound organic phosphates.

2. Bovine hemoglobin devoid of free and loosely bound organic phosphates.

3. Bovine hemoglobin devoid of free and loosely bound organic phosphates, added with 2,3-DPG in a ratio of 2 molecules 2,3-DPG per one hemoglobin-tetramer.

Distinction between free, loosely- and formly-bound phosphates followed that of K l i n g e r et al. [4]. Preparation of hemoglobin devoid of free- and loosely-bound organic phos-

phates was performed according to [4]. Bovine hemoglobin, obtained by the method of Drabkin was applied to a column filled with Dowex 1X8 Cl^-, 200-400 mesh Serva. The eluted Hb is devoid of about 97% free- and loosely-bound organic phosphates.

Oxygen dissociation curves were determined according to A s a k u r a et al. [1]. The measurements were performed on hemoglobin solutions in 0.2 M phosphate buffer, pH 7.0. As a reducing agent $NaBH_4$ was used. From the obtained data, parameters of a resulting straight line were calculated by the method of least squares, since the function

$$\log \frac{y}{1-y} = f \log pO_2$$

is a straight line, of a form: $y = ax + b$, where a is equal to the interaction coefficient n, $b = \log K$, and

$$K = \frac{Hb_n O_{2n}}{Hb_n O_2^n}$$

Ten oxygen dissociation curves were obtained for each kind of hemolysate i.e. 10 for hemoglobin containing free, loosely- and firmly-bound organic phosphates, 10 for hemoglobin devoid of free- and loosely-bound organic phosphates, and 10 for hemoglobin devoid of free- and loosely-bound organic phosphates, added with 2,3-DPG, in a ratio of 2 molecules 2,3-DPG per one hemoglobin tetramer,

E s t i m a t i o n o f 2,3-DPG a c c o r d i n g t o B a r t l e t t [2]. Principle: 2,3-DPG in a medium of concentrated H_2SO_4 forms a colour complex with chromotropic (4,5--dihydroxy-naphthalene-3,6-disulfonic) acid. Absorbance of the complex is measured at 695 nm.

Procedure: 4 ml of 0.01% chromatropic acid is added with 0.02 ml of 1 a sample and heated in a water bath for 2 h. In the presence of 2,3-DPG the solution develops a blue-greenish colour.

For isolation of pure α - and β-chains of bovine globin the method of Clegg was followed, with minor modifications. This method if based on elution of the protein adsorbed on CM-32 cellulose with an increasing continous, linear concentration gradient of sodium ions. Then absorbance of collected fractions is measured at 280 nm. Profile of such chromatographic separation is shown elsewhere ([3], Fig. 2).

RESULTS AND DISCUSSION

Samples stored for 96 h showed distinct increases of absorbance with respect to samples stored for shorter time intervals. A similar phenomenon i.e. an apparent increase of the content of α - and β -chains after 96 h of storage, was demonstrated previously ([3], Table 2). Since that may be due to denaturation changes only, in this paper we limited ourselves to presentation of results obtained after 24, 48 and 72 h of storage. From ab-

Table 1

The content of α-and β-chains (in mg, calculated from absorbance measurements, assuming that the content of α-chains after 24-h storage amounts to 30 mg)

Zawartość łańcuchów α i β (w mg obliczona na podstawie pomiarów absorbancji przy założeniu, że zawartość łańcucha α po 24 godzinach przechowywania wynosi 30 mg)

Содержание цепей α и β (в мг подсчитана на основании абсорбанции при условии, что содержание цепей α после 24 часов хранения насчитывает 30 мг)

Time of storage (h)	Control samples (without 2,3-DPG)			Samples containing 2,3-DPG		
	α + β	α	β	α + β	α	β
24	43.05	30.00	13.05	39.64	26.24	13.40
		s = 4.96	s = 3.07		s = 2.25	s = 3.55
		ε = 2.86	ε = 1.77		ε = 1.01	ε = 1.58
		v = 16.5%	v = 23.5%		v = 8.6%	v = 26.4%
48	41.93	29.28	12.64	38.51	25.25	13.26
		s = 1.82	s = 1.06		s = 3.12	s = 1.61
		ε = 1.05	ε = 0.61		ε = 1.40	ε = 0.72
		v = 6.2%	v = 8.4%		v = 12.4%	v = 12.1%
72	33.35	21.67	11.68	36.80	24.45	12.35
		s = 2.03	s = 2.06		s = 1.73	s = 1.92
		ε = 1.17	ε = 1.19		ε = 0.77	ε = 0.85
		v = 9.4%	v = 17.6%		v = 3.2%	v = 15.5%

s - standard deviation, ε - standard error of arithmetic mean, v - variability correlation coefficient.

sorbance values, the content of α- and β-chains were calculated. Appropriate arithmetic means, standard deviations and standard errors of arithmetic means (expressed in mg globin) as well as variability correlation coefficients are shown in Table 1. These calculations were based on the assumption that the content of α-chain does not change with respect to the initial content after 24 h incubation and is equal to 30 mg.

Table 2

The content of α-and β-chains (in control samples without 2,3-DPG and in samples containing 2,3-DPG) and differences between the samples

Zawartość łańcuchów α i β (w próbach kontrolnych bez 2,3-DPG i próbach z dodatkiem 2,3-DPG) oraz różnice występujące między nimi

Содержание цепей α и β (в контрольных пробах без 2,3-ди-ф-глицерата и пробах с добавкой 2,3-ди-ф-глицерата) а также существующие между ними размечие

Time of storage (h)	α chain			β chain		
	$\bar{X}_k$	$\bar{X}_{DPG}$	$\bar{X}_k - \bar{X}_{DPG}$	$\bar{X}_k$	$\bar{X}_{DPG}$	$\bar{X}_k - \bar{X}_{DPG}$
24	30.00	26.24	3.76	13.05	13.40	-0.35
48	29.28	25.25	4.03	12.64	13.26	-0.62
72	21.76	24.45	-2.78	11.68	12.35	-0.67

$\bar{X}_k$ - control samples, $\bar{X}_{DPG}$ - samples containing 2,3-DPG.

It results from the data shown in Table 2 of the preceding paper [3] that in both sets of experiments, where the initial content of globin was equal to 60 mg in a sample, the content of α-chain decreased down to about 20-25 mg, and the content of β-chain down to about 12-13 mg after 72 h storage at 4°C.

Mean content of α- and β-chains in control samples (without 2,3-DPG) and in samples containing 2,3-DPG is shown in Table 2 and in Fig. 1.

2,3-DPG has a slightly stabilizing effect on the α-chain only after 72 h storage, while after shorter times it seems to accelerate its degradation. Similar results had been obtained previously for ATP (Table 2 of the preceding paper [3]). A

slight stabilizing effect of 2,3-DPG on the β-chain was detectable after 24, 48 and 72 h, but the differences in the protein content between control samples and samples containing 2,3-DPG are not statistically significant. This effect is considerably smaller than the distinct stabilizing action of ATP.

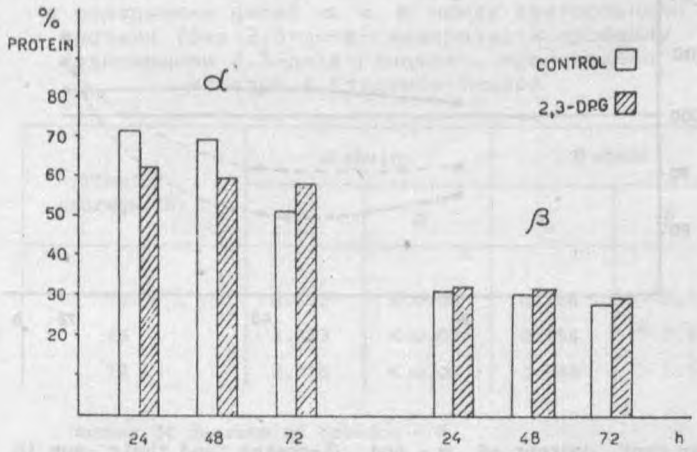

Fig. 1. Effect of 2,3-DPG on the content of globin α- and β-chains (sum of the contents of α- and β-chains was assumed as 100%)

Wpływ 2,3-DPG na zawartość łańcuchów α i β globiny (sumę zawartości łańcuchów α i β przyjęto za 100%)

Влияние 2,3-ди-ф-глицерата на содержание цепей α и β глобина (сумму содержания цепей α и β принято считать за 100%)

Per cent content of α- and β-chains and of their sum with respect to control samples (without 2,3-DPG) is shown in Fig. 2. Respective protein contents in control samples were assumed as 100%.

Statistical significance of the differences in the content of α- and β-chains between control samples (without 2,3-DPG) and samples containing 2,3-DPG was estimated using the Student-Fisher test. Results of these estimations are summarized in Table 3.

The differences in the content of β-chain are not statistically significant while in the case of α-chain are statistically significant for all times of storage.

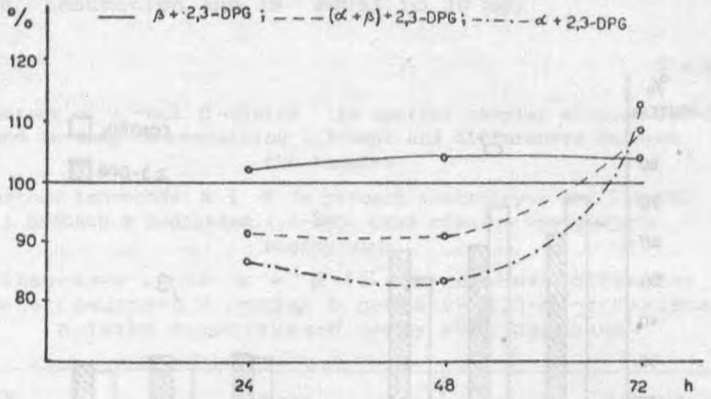

Fig. 2. Per cent content of α- and β-chains and their sum in samples containing 2,3-DPG as compared with control samples (without 2,3-DPG) assumed as 100%

Zawartość procentowa łańcuchów α i β oraz ich sumy w próbach z dodatkiem 2,3-DPG, w stosunku do prób kontrolnych (bez 2,3-DPG), przyjętych za 100%

Процентное содержание цепей α и β а также их суммы в пробах с добавлением 2,3-ди-ф-глицерата по отношению к контрольным пробам (без 2,3-ди-ф-глицерата), принятых за 100%

It is necessary to obtain possibly equivalent amounts of native apo-α and apo-β chains for further studies on the radioprotective effect of hem on the primary structure of hemoglobin.

Table 3

Statistical significance of differences in the content of α- and β-chains between control samples (without 2,3-DPG) and samples containing 2,3-DPG, from the Student-Fisher *t* test

Sprawdzenie istotności statystycznej różnic występujących w zawartości łańcuchów α i β pomiędzy próbami kontrolnymi (bez 2,3-DPG) a próbami zawierającymi 2,3-DPG, wykonane testem *t* Studenta-Fishera

Проверка статистической сущности различий в содержании цепей α и β между контрольными пробами (без 2,3-ди-ф-глицерата) и пробами, содержащими 2,3-ди-ф-глицерат, произведена методом *t* Студента-Фишера

Time of storage (h)	α chain		β chain	
	t_o	p	t_o	p
24	2.593	< 0.05	0.266	> 0.7
48	3.633	< 0.02	0.754	> 0.4
72	2.718	< 0.05	0.648	> 0.5

Number of degrees of freedom = 6.

As apo-β chain is considerably less stable than apo-α chain, utilization of ATP is more suitable than 2,3-DPG is more promising for improvement of the yield of isolated chains. As it was already mentioned [3], the content of DPG is considerably lower in bovine than in human erythrocytes (6 times according to our data, some authors report a lack of this compound in bovine red cells). Therefore 2,3-DPG cannot affect the process of oxygen binding by hemoglobin in vivo in the case of bovine erythrocytes unlike in human red cells.

In order to check whether the added 2,3-DPG binds to globin chains, its content in control samples (without 2,3-DPG) and in samples containing 2,3-DPG separated according to the method of Clegg et al. (chromatographic profile like that shown in Fig. 2 in the preceding paper [3]) was determined by the method

of Bartlett [2]. The estimations were performed for first 10 fractions (non-globin protein) and for fractions containing apo-α and apo-β chains. Samples added with 2,3-DPG showed a considerable level of this ester in initial 10 fractions and small its amounts in fractions containing globin chains. It was not possible to detect any preferentialbinding of 2,3-DPG to any of the isolated proteins by the applied method.

Table 4

Oxygen affinity parameters p_{50} and n of bovine hemoglobin for three different hemolysates

Parametry p_{50} i n powinowactwa tlenowego hemoglobiny wołowej dla trzech różnych hemolizatów

Параметры p_{50} и n кислородного сродства бычьего гемоглобина для трех разных глицератов

Hb containing free and loosely and firmly bound organic phosphates		Hb devoid of free and loosely bound organic phosphates		Hb devoid of free and loosely bound organic phosphates + 2,3-DPG	
p_{50} [mm Hg]	n	p_{50} [mm Hg]	n	p_{50} [mm Hg]	n
21.7	2.25	20.6	2.10	22.8	2.03
21.6	2.26	20.5	2.30	22.5	2.18
21.2	2.30	20.4	2.27	22.3	2.34
20.7	2.32	20.4	2.00	22.3	1.99
20.6	2.45	20.1	2.13	22.0	2.20
20.4	2.29	19.8	2.31	21.9	2.32
20.2	2.21	19.8	2.18	21.7	2.01
20.0	2.39	19.7	1.97	21.4	2.34
19.9	2.12	19.5	2.06	21.3	2.18
19.9	2.40	19.3	2.26	21.1	2.12
$\bar{X}$ p_{50}	s	$\bar{X}$ p_{50}	s	$\bar{X}$ p_{50}	s
20.6	0.7	20.0	0.4	21.9	0.5
$\bar{X}$ n	s	$\bar{X}$ n	s	$\bar{X}$ n	s
2.31	0.08	2.16	0.13	2.17	0.13

Results of measurements of oxygen dissociation curves for hemolysates of different content of phosphate esters are summarized in Table 4.

Table 4 contains also mean values and standard deviations of p_{50} and interaction coefficient n for three kinds of hemolysates.

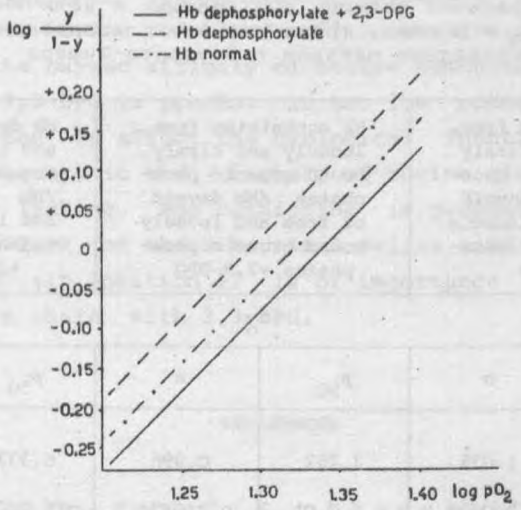

Fig. 3. Oxygen dissociation curves of bovine hemoglobin for three different hemolysates

Krzywe dysocjacji hemoglobiny wołowej dla trzech różnych hemolizatów

Кривые дисоциации бычьего гемоглобина для трех разных глицератов

Figure 3 shows oxygen dissociation curves of bovine hemoglobin for three kinds of hemolysates. The curves are based on mean values of p_{50} and n in a system

$$\log \frac{y}{1-y} = f(\log p_{50})$$

Table 5 contains comparisons of significance of differences in the p_{50} and n values between 3 kinds of hemolysates, using the Student-Fisher t test.

Table 5

Comparison of differences in the values of p_{50} and n between three different hemolysates, by means of the Student-Fisher t test

Porównanie różnic występujących w wartościach p_{50} i n pomiędzy trzema różnymi hemolizatami, wykonane testem t Studenta-Fishera

Сравнение разниц, выступающих в величинах p_{50} и n между трема разными глицератами, проведена методом t Студента-Фишера

Hb containing free, loosely and firmly bound organic phosphates /Hb devoid of free and loosely bound organic phosphates		Hb containing free, loosely and firmly bound organic phosphates /Hb devoid of free and loosely bound organic phosphates +2,3-DPG		Hb devoid of free and loosely bound organic phosphates /Hb devoid of free and loosely bound organic phosphates +2,3-DPG	
t_o					
p_{50}	n	p_{50}	n	p_{50}	n
1.809	1.035	3.752	0.996	6.333	0.062
$p > 0.05$	$p > 0.3$	$p < 0.01$	$p > 0.3$	$p < 0.001$	$p > 0.9$

Number of degrees of freedom = 18.

The obtained results indicate that removal of free and loosely bound organic phosphates does not affect significantly the oxygen affinity of bovine hemoglobin. The addition od 2,3-DPG in a concentration exceeding tenfold physiological values, applied in the present study, results in a considerable decrease of oxygen affinity.

CONCLUSIONS

1. β-chains of bovine globin exhibit a lower stability than α-chains, what is compatible with the data obtained previously in studies of the effect of ATP on the stability of bovine globin chains.

2. Addition of 2,3-DPG does not affect the stability of β-chain so exhibiting a different action than ATP. As it was found previously ATP stabilizes β-chain, too.

3. Fraction of free and loosely bound phosphates does not affect significantly the oxygen affinity of bovine hemoglobin.

4. Addition of 2,3-DPG in a ratio of 2 molecules 2,3-DPG per 1 hemoglobin tetramer (concentration exceeding tenfold the phosphate level in bovine red blood cells) results in a significant decrease of the oxygen affinity of bovine hemoglobin.

However, 2,3-DPG is present in too low concentration in bovine erythrocytes to affect the biological function of hemoglobin in vivo. Moreover, in the β-chain of bovine globin, valine in position 1 is lacking, and methionine is present in position 2. Therefore the lack of the N-terminal valine and of the subsequent histidine (in position 2) is of importance for the interaction of this chain with 2,3-DPG.

REFERENCES

[1] Asakura T., Kawai Y., Yoneyama Y., Yoshikawa H., Anal. Biochem. 7, 393 (1964).

[2] Bartlett G. R., J. Biol. Chem. 234, 469 (1959).

[3] Duda W., Kralisz U., Leyko W., Zesz. Nauk. UŁ, S. II, 23 (1980).

[4] Klinger R., Zahn D., Brox D., Frunder H., Eur. J. Biochem. 18, 171 (1971).

Maszynopis przyjęto do redakcji
28 XII 1977 r.

Department of Biophysics
Institute of Biochemistry
and Biophysics UŁ

Wirgiliusz Duda, Zbigniew Hrabec, Wanda Leyko

WPŁYW 2,3-DPG NA TRWAŁOŚĆ IZOLOWANYCH APO-α i APO-β ŁAŃCUCHÓW HEMOGLOBINY WOŁOWEJ

Uzyskane wyniki świadczą, że oderwanie wolnych i słabo związanych fosforanów organicznych nie wpływa w istotny sposób na powinowactwo tlenowe Hb wo-

łowej. Zastosowany w pracy dodatek 2,3-DPG, dziesięciokrotnie przekraczający wartości fizjologiczne, powoduje wówczas spadek powinowactwa tlenowego.

Łańcuchy β-globiny wołowej wykazują mniejszą trwałość niż łańcuchy α--globiny. Dodatek 2,3-DPG nie wywiera wpływu na trwałość łańcucha β-hemoglobiny wołowej, ma więc inne działanie niż ATP, który jak stwierdzono w poprzedniej pracy, ma działanie stabilizujące na ten łańcuch.

Виргилюш Дуда, Збигнев Храбец, Ванда Лейко

ВЛИЯНИЕ 2,3-ди-ф-ГЛИЦЕРАТА НА ПРОЧНОСТЬ ИЗОЛИРОВАННЫХ АПО-α И АПО-β ЦЕПЕЙ ГЕМОГЛОБИНА БЫЧЬЕЙ КРОВИ

Растворы глобина хранились в присутствии 2,3-ди-ф-глицерата при концентрации 2,3-ди-ф-глицерата : тетрамер Hb = 2 : 1 в деионизированной, 8 М мочевине, с добавлением β-меркаптоэтанола, т = = +4°С. При таких же условиях хранились контрольные пробы, т.е. растворы глобина без 2,3-ди-фГ. Разделение глобинов α и β проводили методом Клегга на колонках с СМ-32 целлюлозой, определяя содержание цепей α и β после 24, 48 и 72 часов хранения.

Полученные результаты свидетельствуют о том, что отсоединение свободных и слабо-связанных органических фосфатов не влияет существенно на сродство к кислороду бычьего гемоглобина. Добавление 2,3-ди-ф-глицерофосфата, в 10 раз превышающее его физиологическое содержание вызывает уменьшение сродства к кислороду.

Цепи β бычьего гемоглобина характеризуются пониженной стабильностью по отношению к α-цепям гемоглобина. Добавление 2,3--ди-ф-глицерофосфата не влияет на стабильность β-цепи бычьего гемоглобина. Так 2,3-ди-ф-глицерофосфат действует по другому чем АТФ, каторый стабилизирует эту цепь, что показано в предыдущей статье.